HYPOXIA-INDUCIBLE FACTORS ENHANCE GLUTAMATE SIGNALING IN CANCER CELLS

by

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Abstract

Hypoxia-inducible factors (HIFs) are transcription factors that are essential in many developmental, physiological and pathophysiological processes. HIFs can be induced in response to reduced oxygen availability (hypoxia) or as a result of oncogene gain-of-function or tumor suppressor gene loss-of-function. Recent studies have shed light on the key roles of HIFs in almost every aspect of cancer biology. On the other hand, the glutamatergic signaling pathway, which was first studied in neuronal excitability in the central nervous system, now has been reported in various human cancers. However, the molecular mechanisms are not fully delineated, leading us to investigate whether HIFs regulate glutamate signaling in cancer.

We show that in hepatocellular and renal carcinoma cells, increased activity of HIFs due to hypoxia or VHL loss-of-function, respectively, augments release of glutamate. After examining the expression of genes which are involved in glutamate transport, we delineated that the glutamate transporters SLC1A1 and SLC1A3 were induced in a HIF-dependent manner. In addition, HIFs coordinately regulate expression of the GRIA2 and GRIA3 genes, which encode α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor subunits, and the expression of FYN, which encodes a SRC family kinase. Binding of glutamate to its cognate receptors activates SRC family kinases and downstream pathways, which stimulate cancer cell proliferation, apoptosis resistance, migration and invasion in a cell-type-specific manner.

Analyzing gene expression data from 42 clear cell renal cell carcinomas (ccRCCs) in the TCGA database revealed that SLC1A1, SLC1A3, GRIA3 and FYN...
mRNA levels were each significantly correlated with the expression of known HIF target genes, indicating that the same transcriptional circuits are active in vivo. Indeed, the AMPA receptor antagonist GYKI 52466 inhibits Hep3B xenograft growth.

This coordinated transcriptional activation of glutamate transporters and receptors by HIFs enhances the glutamate autocrine/paracrine signaling circuit, and is sufficient to activate key signal transduction pathways that promote cancer progression.

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Preface

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Chapter 1

Introduction

$O_2$

Earth is believed to have formed about 4.5 billion years ago whereas oxygen ($O_2$), which we breathe in every second without noticing it, began to rise to appreciable concentrations between 2.5 and 2.0 billion years ago. This shift from an anoxic to an oxic atmosphere is known as the great oxidation event (GOE). In atmospheric evolution, the big question is from when to why: why oxygen levels rose when they did? Despite controversies, oxygenic photosynthesis was the most significant biological event in the history of the Earth’s atmosphere and the evolution of oxygen-producing cyanobacteria represented the most remarkable event in the history of life after the evolution of life.
itself (Kump, 2008). The accumulation of oxygen in the atmosphere forever changed the Earth, making it so diverse and beautiful the way it is today (Canfield, 2005).

The current atmosphere contains (in dry air; by volume) 78.08% Nitrogen ($N_2$), 20.95% $O_2$, 0.93% Argon (Ar), 0.04% Carbon dioxide ($CO_2$) and other trace gases. The average sea-level atmospheric pressure is 760 millimeters of mercury (mmHg) and the partial pressure of oxygen ($PO_2$) in the air at sea level is 21% of 760 mmHg, or 160 mmHg. However, the $O_2$ level to which cells in a multicellular organism are exposed varies considerably. In the human body, it can vary from ~21% or 160 mmHg to less than 1% or 7.6 mmHg. $O_2$ enters the body as room air in the upper airway, while $PO_2$ drops to about 100 mmHg in alveoli. In the alveolar capillaries where blood has just returned from the systemic circulation, the $PO_2$ is only about 40 mmHg and the diffusion of gasses occurs: oxygen diffuses from the alveoli into the blood and carbon dioxide from the blood into the alveoli. Fresh blood with oxygen then flows into the left atrium, left ventricle of the heart and into the arteries, arterioles and into the capillaries, where diffusion occurs between blood and interstitial fluid. Thus there is conceivable variation in $PO_2$ within and between organs and cells can experience low oxygen even under physiological conditions depending on the proximity to blood vessels, tissue architecture and rate of oxygen consumption. Indeed, at the corticomedullary junction of the kidney, $PO_2$ can be less than 10 mmHg (Semenza, 2010b).

*Cellular $O_2$-sensing pathways*

Reduced oxygen availability (hypoxia) occurs not only at the organ level in adults, but also in the uterine environment where fetal arterial and venous $PO_2$ is usually less
than 30 mmHg (4% to 5% O$_2$) (Okazaki and Maltepe, 2006), and serves as a cue for embryonic morphogenesis (Dunwoodie, 2009).

Over the evolutionary history, metazoan species have developed a number of complex yet orchestrated cellular oxygen-sensing pathways in response to low oxygen, including: the metabolic signal integrator and nutrient sensor -- mammalian target of rapamycin (mTOR) kinase; the transcriptional and translational changes resulting from endoplasmic reticulum (ER) stress -- the unfolded protein response (UPR); and the transcription factors -- hypoxia-inducible factors (HIFs) (Figure 1-1).

**Figure 1-1. Cellular O$_2$-sensing pathways.** Three main O$_2$-sensing pathways promote hypoxia tolerance by regulating transcription and mRNA translation. (Wouters and Koritzinsky, 2008).
**Hypoxia-inducible factors (HIFs)**

Among these pathways, hypoxia-inducible factors (HIFs) have established central roles in regulating oxygen homeostasis since the purification and molecular cloning in 1995 (Wang and Semenza, 1995). HIFs are heterodimers, composed of an oxygen-regulated HIF-1α or HIF-2α subunit and a constitutively expressed HIF-1β subunit, which all contain basic helix-loop-helix-PER-ARNT-SIM (bHLH-PAS) domains in the NH2-terminal half (Wang et al., 1995). The COOH-terminal half of HIF-1α and HIF-2α contain two transactivation domains (TADs): TAD-N and TAD-C, representing the NH2-terminal and COOH-terminal transactivation domain respectively (Figure 1-2).

![Diagram](image)

**Figure 1-2. Domain structure of hypoxia-inducible factor (HIF) subunits.** (Prabhakar and Semenza, 2012)
Specific proline (P) residues in the HIF-1α or HIF-2α O2-dependent degradation domain (ODDD) are subjected to O2 dependent hydroxylation by prolyl-4-hydroxylase domain proteins (PHDs) (Kaelin and Ratcliffe, 2008; Keith et al., 2012; Prabhakar and Semenza, 2012). These proline residues, P402 and P564 of human HIF-1α or P405 and P531 of human HIF-2α, are in a conserved core LXXLAP motif (L for leucine, A for alanine, P for proline, X for any amino acid) (Epstein et al., 2001). Three PHDs, PHD1, PHD2 and PHD3, which are encoded by the EGLN2, EGLN1 and EGLN3 genes, respectively, have been identified to date in mammalian cells. PHDs belong to the Fe2+ and 2-oxoglutarate (α-ketoglutarate) dependent dioxygenase superfamily and the reaction that they catalyze is represented as follows:

\[
\text{PHD-Fe}^{2+} + \text{HIF-proline} + 2\text{-OG} + \text{O}_2 \rightarrow \text{PHD-Fe}^{3+} + \text{HIF-4-hydroxy-proline} + \text{Suc} + \text{CO}_2
\]

2-OG stands for 2-oxoglutarate and Suc stands for succinate. Ascorbate is also required, for it reduces Fe3+ to Fe2+.

A hydroxylated proline residue on HIF is required for interaction with the von Hippel-Lindau tumor suppressor protein (VHL), which is the substrate-specific component of an E3 ubiquitin ligase, containing elongins B and C and Cul 2, that targets hydroxylated HIF-1α or HIF-2α for ubiquitination and subsequent proteasomal degradation (Maxwell et al., 1999).

In addition to regulating protein stability of HIFs, O2 also regulates HIF transcriptional activity, which is mediated by an asparaginyl hydroxylase, factor inhibiting HIF-1 (FIH-1) (Mahon et al., 2001). FIH-1, which is also an Fe2+ and 2-oxoglutarate dependent dioxygenase, hydroxylates a conserved asparagine (N) residue located in the TAD-C, N803 of HIF-1α and N847 of HIF-2α, which in turn blocks the
recruitment of the transcriptional coactivators p300 and CREB binding protein (CBP) (Lando et al., 2002).

Under hypoxic conditions when O$_2$, the substrate of PHDs, becomes limited, activities of PHDs are suppressed and this leads to HIF-1$\alpha$ and/or HIF-2$\alpha$ stabilization and accumulation. After dimerization with HIF-1$\beta$ and translocation to the nucleus, HIFs bind to the consensus DNA sequence 5’-RCGTG-3’, which is embedded within hypoxia response elements (HREs) in target genes, and activate their transcription (Semenza et al., 1996). More than 100 genes have been shown as HIF targets, which are involved in metabolic reprogramming, erythropoiesis, angiogenesis and many other adaptive responses to hypoxia.

The above mechanism represents the canonical O$_2$-dependent regulation of HIFs by hydroxylases, but emerging reports have shown that there are also multiple O$_2$-independent pathways regulating HIFs, for example, through other post-transcriptional modifications of HIFs protein, through effects on PHDs activity and through direct interaction with HIFs (Prabhakar and Semenza, 2012).

As the master regulators of oxygen homeostasis, HIFs have been implicated in developmental processes such as mammalian placentation, cardiovascular and bone morphogenesis, branching in tracheal development, cardiovascular-pulmonary development (Simon and Keith, 2008); physiological processes such as neonatal adaptation to umbilical cord compression and hypoxia during birth, O$_2$ sensing by the carotid body and evolutionary adaptation to high altitude among Tibetan and Andean populations (Prabhakar and Semenza, 2012); as well as in pathophysiological processes
such as coronary artery disease and peripheral arterial disease, hereditary erythrocytosis, inflammation, obstructive sleep apnea and solid tumors (Semenza, 2012a).

**Tumor hypoxia**

In 1955, Thomlinson and Gray made the pioneering observation that in human lung squamous carcinoma histology sections, tumors preserved a cord structure with a less than 180-\(\mu\)m-thick sheath consisting of cancer cells with necrosis in the center and stroma outside, and suggested the implications in radiotherapy. They proposed that this resulted from peripheral proliferating tumor cords with no capillaries and consuming nutrients inwards from the immediately surrounding vascularized stroma, which was consistent with the calculated maximum 150-\(\mu\)m radius of a cord with complete anoxia. They proposed that there existed a falling oxygen gradient between the periphery and the tumor cord center and that might have an important bearing on radiotherapy (Thomlinson and Gray, 1955). About 10 years later, Churchill-Davidson reported that cancer patients treated with radiation in combination with hyperbaric \(\text{O}_2\) had an overall better response (Churchill-Davidson, 1964).

The early studies were encouraging, yet the focus was on tissue oxygenation and radioresistance (Figure 1-3). However, it was not until the purification and cloning of HIFs that the direct cellular responses to hypoxia were determined.
HIFs in Cancer

Following its purification, HIFs have been extensively explored in various fields including cancer. Accumulating reports have established key roles of HIFs in many critical aspects of cancer biology, including metabolic reprogramming, proliferation, survival, stem cell maintenance, angiogenesis, epithelial-mesenchymal transition, immune evasion, invasion, metastasis, and resistance to chemotherapy and radiation therapy (Semenza, 2010a) (Figure 1-4).
Regions of intratumoral hypoxia are present in many solid cancers, leading to induction of HIF activity (Harris, 2002). In addition, oncogene gain-of-function or tumor suppressor gene loss-of-function also stimulates increased HIF activity in cancer (Semenza, 2003). The most notable example of a genetic alteration driving HIF activity is in clear cell Renal Cell Carcinoma (ccRCC), which is characterized by a high incidence of VHL loss-of-function due to either mutation or epigenetic silencing, which leads to high HIF activity even under non-hypoxic conditions (Network, 2013). High levels of HIF-1α or HIF-2α in the diagnostic biopsy have been shown to be associated with
metastasis, treatment failure, and patient mortality in many types of cancer (Semenza, 2003; Semenza, 2010a).

Glutamate

Glutamic acid is one of the non-essential proteinogenic amino acids. It was first discovered and identified in 1866 by the German chemist Karl Ritthausen from gliadin, a component of wheat gluten. In 1908, a Japanese professor of the Tokyo Imperial University, Kikunae Ikeda, obtained 30 grams of (S)-glutamic acid from 40 kilograms of the kombu seaweed. He identified (S)-glutamic acid as the taste-enhancing component and named this flavor umami. The isolation of a crystalline salt of glutamic acid, monosodium glutamate (MSG), from wheat flour was patented by Ikeda, and in 1909, the first MSG was mass-produced commercially under the trade name Ajinomoto (The meaning of “Aji no Moto” is “Essence of Taste”). Since then, MSG has been used worldwide as an additive or seasoning to enhance the flavor. The structure of glutamic acid, glutamate and monosodium glutamate (MSG) is shown (Figure 1-5).

![Structure of glutamic acid, glutamate and monosodium glutamate](image)

Figure 1-5. Structure of glutamic acid, glutamate and monosodium glutamate.
L-Glutamate is the most abundant intracellular amino acid (the reported concentrations vary between 2 and 20 mM), while the extracellular concentration in vivo is very low, approximately 20 μM. L-Glutamate is at the crossroads of many important metabolism pathways, where it can be converted into γ-amino butyric acid (GABA), glucose, glutathione, 2-oxoglutarate, or ornithine (Figure 1-6).

**Figure 1-6. Glutamate is a key metabolite in mammalian cells.** The probable functions of the glutamate products are indicated, as well as the cells or organs where the metabolic pathway preferentially occurs. glutamate Dh, glutamate dehydrogenase; NO, nitric oxide; iNOS, inducible nitric oxide synthase. (Newsholme et al., 2003)
Of all the processes glutamate has participated in, transamination is of special attention since it is an important step to synthesize some non-essential amino acids.

Examples are as follows:

\[
\text{pyruvate + glutamate } \rightleftharpoons \text{alanine + } \alpha\text{-ketoglutarate}
\]

This reaction is catalyzed by glutamate pyruvate transaminase (GPT), which is also known as alanine transaminase/aminotransferase (ALT).

\[
\text{oxaloacetate + glutamate } \rightleftharpoons \text{aspartate + } \alpha\text{-ketoglutarate}
\]

This reaction is catalyzed by glutamate oxaloacetate transaminase (GOT), which is also known as aspartate transaminase/aminotransferase (AST).

**Glutamate Signaling in CNS**

Binding of the neurotransmitters could affect the postsynaptic neuron in either an excitatory or inhibitory manner. The major excitatory transmitters at synapses in the central nervous system (CNS) are glutamate and acetylcholine and the major inhibitory transmitters are glycine and \(\gamma\)-amino butyric acid. Glutamate is the most abundant excitatory neurotransmitter of CNS.

Glutamate acts on two classes of cell surface receptors, which are designated metabotropic and ionotropic (Figure 1-7). The metabotropic receptors are G protein-coupled receptors that are encoded by the \(GRM1-8\) genes. The ionotropic receptors include three subclasses: \(N\)-methyl-D-aspartate (NMDA) receptors, which are encoded by the \(GRIN1, GRIN2A-D,\) and \(GRIN3A-B\) genes; \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which are encoded by the \(GRIA1-4\) genes; and kainate receptors, which are encoded by the \(GRIK1-5\) genes (Meldrum, 2000).
Figure 1-7. The glutamate receptors and their primary signal transduction mechanism. Upon glutamate binding and activation, the ionotropic receptors are Ca\textsuperscript{2+} and/or Na\textsuperscript{+} permeable; while the metabotropic receptors coupled G proteins either activate downstream phospholipase C (PLC) or inhibit adenylate cyclase (AC). (Kew and Kemp, 2005)

Glutamate is stored in vesicles at the synapses. Upon activation, the pre-synaptic neuron releases glutamate, which binds to and activates the glutamate receptors located on the membranes of post-synaptic neurons. Glutamate receptors that function at the excitatory synapse are implicated shown (Figure 1-8). However, glutamate signaling has now been implicated in a variety of non-excitatory tissues, as well as in several diseases, including cancer (Nedergaard et al., 2002).
Glutamate Signaling in Cancer

It has been reported that there is a correlation between serum glutamate levels and Tumor/Gleason Score in prostate cancer (Figure 1-9).

A role for glutamate signaling in cancer was first reported for glioma, in which glutamate secretion by the cancer cells was shown to induce excitotoxic death of neighboring neurons, thereby facilitating tumor growth and invasion (Takano et al., 2001; Ye and Sontheimer, 1999). Subsequently, glutamate antagonists were shown to inhibit the proliferation and migration of glioma and other cancer cell types (Rzeski et al., 2001).
Figure 1-9. Serum glutamate levels in normal individuals and patients with prostate cancer. There are patients with primary (Primary PCa) or metastatic castrate-resistant prostate cancer (mCRPCa). Patients with primary PCa were divided according to their aggressiveness score based on Gleason and PSA (middle) or according to Gleason score (right). A. Results from the entire study cohorts. B. Results from the entire study cohorts of Caucasian Americans. C. Results from the entire study cohorts of African Americans. The box frame
defines the 25 to 75 percentiles; the whiskers depict the minimum and maximum values; the line within the box marks the median value.

(Koochekpour et al., 2012)

Glutamate receptors have been implicated in several different types of cancer. Insertional mutagenesis of Grml or melanocyte-specific overexpression of Grml or Grm5 led to melanoma in transgenic mouse models (Choi et al., 2011; Pollock et al., 2003). All 24 genes encoding glutamate receptor subunits have been detected at the mRNA level in various cancer cell lines (Stepulak et al., 2009). Molecular and biochemical studies of glutamate receptors have demonstrated their roles in various cancer types (de Groot et al., 2008; Luksch et al., 2011; Namkoong et al., 2007; Watanabe et al., 2008). Recent high-throughput genomic studies have identified GRM1, GRM3, GRM4, GRM8 and GRIN2A as susceptibility genes in non-small-cell lung cancer (NSCLC), melanoma, osteosarcoma, and bladder cancer (Kan et al., 2010; Morrison et al., 2014; Prickett et al., 2011; Savage et al., 2013; Wei et al., 2011). In contrast, GRIK2, which is the gene most frequently involved in deletions of chromosome 6q in acute lymphocytic leukemia, is regarded as a tumor suppressor gene (Sinclair et al., 2004). Additionally, hypermethylation of GRIK1, GRIK2, GRIN2A and GRIN2B has been reported in ccRCC, gastric cancer, colon cancer, esophageal squamous cell carcinoma and NSCLC (Ibragimova et al., 2013; Kim et al., 2008; Kim et al., 2007; Liu et al., 2007; Tamura et al., 2011; Wu et al., 2010). Thus, the effect of gain or loss of glutamate receptor function is cancer type specific.

In this thesis, we demonstrate that HIF activity, induced by hypoxia or VHL loss-of-function in hepatocellular and renal carcinoma cells, respectively, mediates the
coordinate transcription of multiple genes encoding glutamate transporters and glutamate receptors, which results in activation of signal transduction pathways that stimulate cancer cell proliferation, survival, or migration and invasion in a cancer cell-type-specific manner. Our results demonstrate that HIFs mediate autocrine/paracrine glutamate signaling that promotes cancer progression.
Chapter 2

Materials & Methods

Cell Culture and Reagents

Hepatocellular carcinoma cell line Hep3B; Clear cell renal cell carcinoma cell lines 786-O, RCC4; Breast cancer cell lines MDA-MB-231, MDA-MB-435; and Human Embryonic Kidney cells HEK293T were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin (complete medium).

To establish stable knockdown cell lines, recombinant lentivirus was generated by transfection of HEK293T cells with the transducing vector pLKO.1-puro encoding shRNA, together with packaging vectors pMD.G and pCMV-dR8.91, using PolyJet (SignaGen). After 48 h, medium containing viral particles was harvested and passed
through a 0.45-μm filter (Millipore). Hep3B cells were transduced with viral supernatant in the presence of 8 μg/mL of Polybrene (Sigma-Aldrich). After 24 h, cells were replenished with fresh medium containing 2 μg/mL of puromycin. Cells were maintained in puromycin-containing medium for selection of stable transfectants. shRNA sequences are listed in Table 2-1.

Table 2-1. **Nucleotide sequence of shRNAs.** The table provides the nucleotide sequence of the shRNA constructs used to generate lentivirus particles and silence expression of HIF-1α, HIF-2α, and FYN.

<table>
<thead>
<tr>
<th>shRNA</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>CCAGTTATGATTGTGAAGTTA</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>GGAGACGGAGGTGTTCTAT</td>
</tr>
<tr>
<td>FYN #1</td>
<td>GCCTATTCACTTTTCTATCCGT</td>
</tr>
<tr>
<td>FYN #2</td>
<td>GTGCCAACAATCCTAGTGCTT</td>
</tr>
<tr>
<td>Non-targeting</td>
<td>CAACAAGATGAAGAGCACCAA</td>
</tr>
</tbody>
</table>

VHL-deficient cell lines 786-O and RCC4 were transfected with expression vector encoding VHL and neomycin resistance. Stable subclones of 786-O-VHL and RCC4-VHL cells were maintained in complete medium with G418 (0.8 mg/mL). Culture conditions for RCC4-shEV, RCC4-sh1α, RCC4-sh2α, and RCC4-sh1α/2α were described previously (Krishnamachary et al., 2006; Zhang et al., 2007). Briefly, short hairpin RNA (shRNA) specific for HIF-1α (5’- GTTACGTTCCTTGCATCAG-3’) was identified and cloned into pSUPER.retro.neo.GFP (OligoEngine, Seattle, WA); shRNA specific for HIF-2α (5’-AGAAGAGTAACCTTCCTATT-3’) were identified and cloned into
pSUPER.retro.puro. RCC4 cells were infected with the HIF-1α shRNA retrovirus and selected with either G418 (RCC4-sh1α). A virus expressing the empty vector of pSUPER.retro.neo.GFP was used as a control (RCC4-shEV). RCC4-sh2α pool was established by retrovirus infection and puromycin selection. RCC4-sh1α/2α was established by HIF-1α shRNA and HIF-2α shRNA retrovirus infection and antibiotic selection with both G418 and puromycin.

All cells were maintained at 37°C in a 5% CO₂/95% air incubator. For hypoxic exposure, cells were placed in a modular incubator chamber (Billups-Rothenberg) flushed with 1%O₂/5% CO₂/balance N₂ and incubated at 37°C.

GYKI 52466, MK-801, Evans Blue and Sulfasalazine were purchased from Sigma-Aldrich. U0126 was purchased from LC laboratories. Saracatinib was purchased from Selleckchem.

Promoter Reporter Plasmid Constructs

The double-stranded 55-bp oligonucleotides encompassing the HRE for each indicated gene were annealed and cloned into luciferase reporter plasmid pGL2-Promoter cut by BamHI and Sall (Promega). All plasmid constructs were confirmed by nucleotide sequencing.

Glutamate measurement

Cells were incubated for indicated time before harvest the medium. Glutamate levels in media were measured using a colorimetric glutamate assay (BioVision) in which
optical density at $\lambda = 450$ nm was determined using a microplate reader (PerkinElmer) and glutamate concentration was interpolated from a standard curve (A representative standard curve was shown, Figure 2-1) and corrected for differences in cell number.

![Glutamate Standard Curve](image)

**Figure 2-1. A representative glutamate standard curve.**

---

**RNA Isolation, Reverse Transcription and Real-time PCR**

Total RNA was extracted using TRIzol (Invitrogen). Briefly, samples were homogenized with Trizol, precipitated with isopropanol, and washed with 70% ethanol.

RNA was then treated with DNase I (Ambion), and reverse transcribed with the iScript cDNA Synthesis kit (Bio-Rad).

qPCR analysis was performed using Maxima SYBR Green Master Mix (Fermentas) with the iCycler Real-time PCR Detection System (BioRad). The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression of genes of interest in all
experimental conditions. Results were normalized to the 18S rRNA signal. Primer sequences are listed in Table 2-2.

**Table 2-2. Nucleotide sequence of primers used for RT-qPCR.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SLC1A1</strong></td>
<td>Forward: CTTGGAATCCACAATCCTTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTGAGGTCTGGGTGAATGAG</td>
</tr>
<tr>
<td><strong>SLC1A2</strong></td>
<td>Forward: AGTGCTGGAACCTTTGCCTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CATCCATGTTAATGGTTGCTC</td>
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<tr>
<td><strong>SLC1A3</strong></td>
<td>Forward: AAACCAAGCGTGAAGAAGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AAGATAATCAGGCCAGGAC</td>
</tr>
<tr>
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<td>Forward: GGTCTGCCCTGAGAAATCCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAGATAAAATCAGCCCAGCA</td>
</tr>
<tr>
<td><strong>GRIA1</strong></td>
<td>Forward: GGTCTGCCCTGAGAAATCCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CTCGCCCTTGTCGTACCAC</td>
</tr>
<tr>
<td><strong>GRIA2</strong></td>
<td>Forward: GTGGCTAGAGTGCGGAAGTC</td>
</tr>
<tr>
<td></td>
<td>Reverse: CACCAACTTTTCATGGTGTCG</td>
</tr>
<tr>
<td><strong>GRIA3</strong></td>
<td>Forward: GGTCTGCCCTGAGAAATCCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAAGCTAGGCCTAACAAGGGAT</td>
</tr>
<tr>
<td><strong>GRIA4</strong></td>
<td>Forward: ATTTGGTGTCAGCGTGTTTCA</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCAGGAAAAACCAGAGGC</td>
</tr>
<tr>
<td><strong>FYN</strong></td>
<td>Forward: GGTCTGCCCTGAGAAATCCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: CATCTTCGTCCGTGCTTCA</td>
</tr>
<tr>
<td><strong>18S rRNA</strong></td>
<td>Forward: CGGCGACGACCCCCATTGAAC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAATCGAACCCTGATTCCCGTC</td>
</tr>
</tbody>
</table>
**Immunoblot Assays**

Cells were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% IGEPAL CA630, 0.1% SDS, 0.25% sodium deoxycholate, 5 mM EDTA, 1 mM NaF, 1 mM Na$_3$VO$_4$ and protease inhibitors), centrifuged for 15 min at 13,000 rpm at 4°C and the insoluble debris were discarded. Whole cell lysates were fractionated by SDS-PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated for 1 hour with blocking buffer containing tris-buffered saline, 5% non-fat dry milk and 0.1% Tween-20, and then incubated overnight with primary antibodies diluted in blocking buffer. The membrane was washed and incubated with corresponding HRP-conjugated secondary antibody for 1 hour. After wash, the membrane was developed using ECL, ECL-plus or ECL-prime reagent (Amersham).

The following primary antibodies were used: HIF-1α (BD Biosciences); HIF-2α, SLC1A1, SLC1A3, GRIA2, GRIA3, FYN, pLYN$^{Y396}$, LYN, SRC, LCK, ERK, Caspase-3, and AKT (Novus Biologicals); and phosphorylated ERK, phosphorylated AKT, and β-actin (Santa Cruz). HRP-conjugated secondary rabbit antibody was purchased from GE Healthcare Life Sciences; all other secondary antibodies were obtained from Santa Cruz.

**Chromatin Immunoprecipitation assays**

Chromatin Immunoprecipitation (ChIP) assays were performed according to ChIP Assay Kit protocol (Upstate). Briefly, Hep3B cells were exposed to 20% O$_2$ or 1% O$_2$ for 24 h before harvest. Then formaldehyde was added directly to the tissue culture media to
a final concentration of 1% and incubated on a shaking platform for 15 min at room temperature. The crosslinking reaction was quenched by adding glycine to a final concentration of 0.125 M and incubated on the shaking platform for 5 min at room temperature. The cells were washed with cold PBS, scraped with cold PBS containing protease inhibitors and centrifuged. The pellet was then resuspended in SDS Lysis Buffer (50 mM Tris (pH 8.1), 10 mM EDTA, 1% SDS and protease inhibitors) and incubated on ice for 10 min. Next, cell lysates were sonicated using a Bioruptor (Diagenode) and centrifuged at 14,000 rpm for 10 min at 4°C. The supernatant was transferred to a new tube, diluted 10 fold in ChIP Dilution Buffer (16.7 mM Tris-HCl (pH 8.1), 167 mM NaCl, 1.2 mM EDTA, 1.1% Triton X-100, 0.01% SDS and protease inhibitors) and precleared with salmon-sperm DNA/protein A agarose slurry (Millipore) by incubating on a rotating platform for 1 h at 4°C. Samples were centrifuged for 5 min at 13,000 rpm at 4°C. The supernatant was transferred to a new tube and aliquoted for immunoprecipitation.

IgG (Santa Cruz and Novus Biologicals) or primary antibody against HIF-1α (Santa Cruz), HIF-2α (Novus Biologicals), or HIF-1β (Novus Biologicals) was incubated with precleared lysates on ice for 1 h. Salmon sperm DNA/protein A agarose slurry was added and incubated on a rotating platform at 4°C overnight. The agarose beads were washed sequentially with: Low Salt Immune Complex wash buffer [20 mM Tris (pH 8.1), 150 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100]; High Salt Immune Complex wash buffer [20 mM Tris (pH 8.1), 500 mM NaCl, 2 mM EDTA, 0.1% SDS and 1% Triton X-100]; LiCl Immune Complex wash buffer [10 mM Tris (pH 8.1), 0.25 M LiCl, 1 mM EDTA, 1% sodium deoxycholate and 1% IGEPAL CA630]; and twice with TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA]. For each wash, samples were rotated
for 3–5 min on a rotating platform at room temperature and centrifuged for 5 min at 1,000 rpm at 4°C.

Chromatin was eluted by adding 250 μL elution buffer (1% SDS and 0.1 M NaHCO₃, freshly prepared), rotated for 15 min on a rotating platform and centrifuged for 2 min at 13,000 rpm at room temperature. The supernatant was transferred to a new tube. The elution process was repeated once and the eluates were combined. 20 μL 5 M NaCl was added and samples were incubated at 65°C for 4 h to reverse formaldehyde crosslinks. Next, 10 μL 0.5 M EDTA, 20 μL 1 M Tris-HCl (pH6.5) and 1 μL proteinase K (Roche) were added to each sample and incubated at 45°C for 1 h to degrade any protein including nucleases.

DNA was purified by phenol-chloroform extraction and ethanol precipitation, and then reconstituted in 50 μL water. DNA samples were added 1 μL of RNase and incubated at 37°C for 1 h. Primer sequences for qPCR were listed in Table 2-3.

**Table 2-3. Nucleotide sequence of primers used for qPCR in ChIP assays.**

<table>
<thead>
<tr>
<th></th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>SLC1A3 HRE</td>
<td>Forward: TAATGGAGCTGCCACCCTAT</td>
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<tr>
<td></td>
<td>Reverse: CAAGCTCCTCCATCTGAAGC</td>
</tr>
<tr>
<td>GRIA2 HRE</td>
<td>Forward: CCGAGCTGTGCTTTTCTCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGAGAGGGGCAGGCAGTC</td>
</tr>
<tr>
<td>FYN HRE#1</td>
<td>Forward: AGCCTATGGCCACAAGTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: GAGCTGGGAGCAAGTGAGAT</td>
</tr>
<tr>
<td>FYN HRE#2</td>
<td>Forward: TTGGAACAAAATTTGGGCAGT</td>
</tr>
<tr>
<td></td>
<td>Reverse: CCAGTGGGTCTGACTGTGG</td>
</tr>
</tbody>
</table>
**Luciferase Reporter Assays**

Hep3B cells were seeded in a 48-well plate. After overnight incubation, cells were co-transfected with pGL2 firefly luciferase reporter plasmid with HRE-WT or HRE-MUT sequences and pSV- Renilla. 24 h after transfection, cells were exposed to 20% or 1% O2 for 24 h and lysed. Luciferase activities were determined with a multi-well luminescence reader (PerkinElmer) using the Dual-Luciferase Reporter Assay System (Promega). Firefly:Renilla luciferase ratio was calculated and normalized to HRE-WT-20% O2.

**Xenograft Assays**

All animal protocols were approved by The Johns Hopkins University Animal Care and Use Committee. Male SCID mice 5 to 7 weeks of age were used. Hep3B cells were resuspended at 2.5 x 10^7 cells/ml in a 1:1 mix of DMEM:Matrigel (BD Biosciences). A 200 μL suspension containing 5 x 10^6 cells was implanted subcutaneously into the right flank. Mice were monitored for body weight and tumor volume (mm³), which was calculated as length (mm) x [width (mm)]^2 x 0.52. When tumor volume reached 200 mm³, mice were randomly divided into control and treatment groups and received daily intraperitoneal injections of vehicle or 5 mg/kg of GYKI 52466, respectively. Mice were euthanized 45 days after tumor implantation. Primary tumors were harvested for hematoxylin and eosin (H&E) staining and immunohistochemistry.
Immunohistochemistry

Xenograft tumors were fixed in 10% formalin, paraffin embedded, and 5 μm sections were prepared. The sections were dewaxed with xylene (3 min x 2), hydrated with a decreasing gradient of ethanol (100% ethanol, 95% ethanol, 80% ethanol and water), followed by antigen retrieval using citrate-EDTA buffer (10 mM Citric Acid (pH 6.1), 2 mM EDTA and 0.05% Tween 20). The slides were placed in a coplin jar containing pre-warmed citrate-EDTA buffer (7~9 min in microwave to ~ 95°C), heated in a rice cooker for 40 min (~ 85°C), cooled down for 30 min and rinsed twice with PBS.

The LSAB+ System HRP kit (DAKO) was used with Ki67 antibody (1:200, Novus Biologicals). Briefly, the slides were incubated in 3% H₂O₂ for 10 min to quench endogenous peroxidase activity; washed sequentially with H₂O and PBS for 5 min; blocked in 1% BSA/PBS for 30 min; washed twice with PBS; and incubated with Ki67 antibody at a 1:200 dilution in 1% BSA/PBS overnight at 4°C. After three times of wash with PBS, slides were incubated with Biotinylated Link at room temperature for 30 min. After three times of wash with PBS, slides were incubated with Streptavidin-HRP at room temperature for 30 min. After another three times of wash with PBS, slides were incubated with substrate-chromogen solution at room temperature for 30 seconds and rinsed gently with distilled water.

Sections were counterstained with Mayer’s hematoxylin for 5 min; rinsed for three times with water; dipped briefly in blueing reagent 0.2% Ammonia Water Solution; rinsed for three times with water. The slides were then dehydrated with an increasing gradient of ethanol (80% ethanol 95% ethanol, and 100% ethanol), followed by xylene (3 min x 2), and mounted with Parmount agent.
The Ki67 staining was quantified using ImageJ software (NIH) (Xiang et al., 2013). Briefly, the acquired images in RGB color were separated into different color channels by a deconvolution method. The Image J plug-in for color deconvolution has a built-in vector for separating hematoxylin and diaminobenzidine (DAB) staining. After color deconvolution, hematoxylin and DAB images were processed separately. By using six to eight random fields per tumor section stained with the antibody of interest, suitable threshold levels for hematoxylin and DAB were determined. These thresholds were used on both hematoxylin and DAB images and kept constant for analysis of the main image dataset. The extent of staining was calculated as the DAB-positive area divided by the hematoxylin-positive area. Six to eight fields, which encompassed the total surface area of each histological section, were analyzed. Ki67 staining were calculated as the number of DAB-stained cells divided by the number of total cells per field.

**Flow Cytometry**

Cultured cells were washed with PBS, harvested by trypsinizing, and resuspended in with 1x Binding Buffer (BD Pharmingen) at a concentration of 1 x 10^6 cells/mL. 100 μL of the solution was transfer to a FACS tube and 5 μL of PE-conjugated Annexin V and 5 μL of 7-amino-actinomycin (7-AAD) were added. The cells were gently vortexed and incubated for 15 min at room temperature in the dark.

400 μL of 1x Binding Buffer was added to each tube and the cell samples were analyzed with a LSR-II flow cytometer (Becton Dickinson).
Migration and invasion assays

For migration assays, RCC4 and RCC4-VHL cells were seeded onto uncoated inserts of a 24-well Transwell chamber (8-mm pore size; Costar) and allowed to migrate for 16 h in the presence of vehicle or 250 μM GYKI 52466, or vehicle or 10 μM Saracatinib.

For invasion assays, Transwell inserts were first coated with Matrigel (BD Biosciences) at 37°C for 4 h and then cells were seeded onto the coated inserts. Cells were allowed to invade for 24 h in the presence of vehicle or 250 μM GYKI 52466, or vehicle or 10 μM Saracatinib.

The cells that remained on the upper surface of the filter were removed with a cotton swab. The cells that migrated/invaded to the underside of the inserts were fixed with 100% methanol for 5 min and stained with 0.1% crystal violet for 20 min. Membranes were cut from the Transwell and rinsed in 1 mL of 33% acetic acid. Absorbance was read at 570 nm.

Correlation analysis of microarray expression data

PROGgene, a gene expression based survival analysis web application for multiple cancers, was used to assess survival data (http://watson.compbio.iupui.edu/chirayu/proggene/database/?url=proggene). GSE10141 and GSE29609 were analyzed for hepatocellular carcinoma and clear cell Renal Cell Carcinoma, respectively.
Gene expression data from Kidney Renal Clear Cell Carcinoma (TCGA) were obtained from the International Cancer Genome Consortium (http://icgc.org/). Pearson’s correlation coefficient was used to determine p values for co-expression.

**Statistical analysis**

Data were presented as mean ± SEM and analyzed with an unpaired two-tailed Student’s t-test or ANOVA followed by Bonferroni post-test as indicated in the figure legend. A p value < 0.05 was considered significant.
Chapter 3

HIF-dependent glutamate signaling in Hep3B cells

Hep3B cells release more glutamate under hypoxia

Human glioma, mouse melanoma, rat prostate cancer, and human breast cancer cells have been shown to release glutamate (Seidlitz et al., 2009; Ye and Sontheimer, 1999). High levels of extracellular glutamate also accumulate in response to cerebral ischemia (Nishizawa, 2001). We hypothesized that hypoxia may induce glutamate release from cancer cells.

Human hepatocellular carcinoma Hep3B cells were employed to assess the effect of hypoxia on glutamate release in cancer cells. The cells were maintained at 20% O₂ or exposed to 1% O₂ for 24 or 48 h before collecting the culture media. Extracellular glutamate concentration was measured. We observed a time-dependent increase of extracellular glutamate in the media of cells exposed to hypoxia, as compared to cells
maintained at 20% O₂ (Figure 3-1). This result suggests that reduced oxygen availability triggers increased glutamate release from Hep3B cells.

![Graph showing time-dependent increase of extracellular glutamate in the media of cells exposed to hypoxia.](image)

**Figure 3-1. Time-dependent increase of extracellular glutamate in the media of cells exposed to hypoxia.** Hep3B cells were exposed to 20% O₂ for 48h, 20% O₂ for 24h+1% O₂ for 24h and 1% O₂ for 48h. Medium were harvested and extracellular glutamate concentration were measured and corrected to cell number in each condition. Shown glutamate levels were normalized to results obtained at 20% O₂ (mean ± SEM; n = 3). **p <0.01 vs. 20% O₂. One-way ANOVA with Bonferroni’s Multiple Comparison Test.

**Hypoxia induces membrane glutamate transporter expression in Hep3B**

There are several molecular mechanisms by which glutamate release is mediated: vesicular glutamate transporters (encoded by genes *SLC17A6-8*); the cystine-glutamate antiporter system x_c⁻ (encoded by *SLC7A11* and *SLC3A2*); and the membrane glutamate
transporters EAAT3, EAAT2, EAAT1, EAAT4, and EAAT5 (encoded by \textit{SLC1A1}, \\
\textit{SLC1A2}, \textit{SLC1A3}, \textit{SLC1A6}, and \textit{SLC1A7}).

We sought to determine which mechanism was responsible for the increased glutamate release under hypoxic conditions. Total RNA was isolated from Hep3B cells that were exposed to 20\% or 1\% O$_2$ for 24 h. Analysis by reverse transcription and quantitative real-time PCR (RT-qPCR) revealed that \textit{SLC1A1} and \textit{SLC1A3} mRNA levels were significantly induced by hypoxia, whereas \textit{SLC1A2} and \textit{SLC7A11} mRNA were not affected (Figure 3-2A). To further confirm the specificity, Hep3B cells were treated with dimethyloxalylglycine (DMOG), which inhibits the PHDs. \textit{SLC1A1} and \textit{SLC1A3} mRNA expression was also increased in response to DMOG (Figure 3-2B). Hypoxic induction of \textit{SLC1A1} and \textit{SLC1A3} mRNA was not observed in two breast cancer cell lines (Figure 3-2C-D), indicating that it was a cell-type-specific response.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Hypoxic induction of \textit{SLC1A1} and \textit{SLC1A3} mRNA.}
\end{figure}
Figure 3-2. Hypoxia induces membrane glutamate transporter expression in Hep3B cells. (A-B) Cells were exposed for 24 h, to 20% or 1% O₂ (A), or to vehicle (DMSO) or 100 μM DMOG (B). mRNAs were analyzed by RT-qPCR and normalized to 20% O₂ or DMSO. *p<0.05, **p<0.01 vs 20% O₂ or DMSO, Student’s t-test. (C-D) Two breast cancer cell lines, MDA-MB-231 and MDA-MB-435, were exposed to 20% or 1% O₂ for 24 h. SLC1A1 (C) and SLC1A3 (D) mRNAs were analyzed by RT-qPCR and normalized to 20% O₂. Data are mean ± SEM from ≥ 3 experiments.

**HIFs mediate SLC1A1 and SLC1A3 gene expression in hypoxic Hep3B cells**

To determine whether HIF-1 or HIF-2 was directly responsible for the hypoxia-induced SLC1A1 and SLC1A3 expression, Hep3B cells were stably transfected with vectors encoding: a short hairpin RNA (shRNA) that was non-targeting (shNT); shRNA targeting HIF-1α (sh1α) or HIF-2α (sh2α); or shRNAs targeting both HIF-1α and HIF-2α (sh1α/2α). The effects of these shRNAs on their targets were confirmed by immunoblot assays (Figure 3-3).
Figure 3-3. HIF knockdown in Hep3B cells. Immunoblot assays were performed using lysates from subclones exposed to 20% or 1% O₂.

Analysis of SLC1A1 (Figure 3-4A) and SLC1A3 (Figure 3-4B) mRNA revealed that hypoxia-induced expression was abrogated when HIF-1α, HIF-2α, or both were knocked down in Hep3B cells.

![Figure 3-3](image)

Figure 3-4. SLC1A1 and SLC1A3 gene expression were dependent on HIFs. (A-B)

SLC1A1 (A) and SLC1A3 (B) mRNA were analyzed by real-time PCR in Hep3B subclones, and exposed to 20% O₂ or 1% O₂ for 24 hours. **p < 0.01, ***p < 0.001 vs. shNT at 20% O₂. ###p < 0.001 vs. shNT at 1% O₂. Two-way ANOVA with Bonferroni post test. (mean ± SEM; n = 4).

SLC1A3 is a direct HIF target gene

To examine whether SLC1A3 was a direct HIF target gene, we searched for the HIF consensus binding site sequence 5’-RCGTG-3’ along the gene and designed primers
flanking these sequences. Chromatin immunoprecipitation (ChIP) assays were then performed using these primers for qPCR assays of immunoprecipitated DNA. One site located 2 kb downstream of the SLC1A3 gene (gray oval in Figure 3-5A) was enriched by immunoprecipitation of chromatin from hypoxic cells with HIF-1α, HIF-2α (Figure 3-5B), or HIF-1β (Figure 3-5C) antibodies.

Figure 3-5. A candidate site compassing HRE of SLC1A3 was enriched. (A)

Schematic view of the SLC1A3 gene. Exons were indicated by the black bars and the candidate HRE was indicated by the grey oval. (B-C) Hep3B cells were exposed to 20% O₂ or 1% O₂ for 24 h. ChIP assay was performed using IgG, HIF-1α, HIF-2α (B) and HIF-1β (C) antibodies. Specific primers flanking SLC1A3 HRE were used for qPCR. **p < 0.05, **p < 0.01 vs. the corresponding antibody at 20% O₂. Bonferroni post test after ANOVA (mean ± SEM; n = 3).
To test whether this HIF site was embedded in an HRE, a 55-bp sequence spanning the site was inserted into the reporter plasmid pGL2-promoter, in which a basal SV40 promoter drives firefly luciferase expression. Hep3B cells were co-transfected with this SLC1A3 HRE reporter and a control pSV-Renilla reporter, in which the basal SV40 promoter alone drives Renilla luciferase expression. Transfected cells were exposed to 20% or 1% O\textsubscript{2} for 24 h. The ratio of firefly:Renilla luciferase activity increased with hypoxic exposure, whereas mutation of the HIF binding site in the SLC1A3 HRE (5’-ACGTG-3’ to 5’-AAAAG-3’) significantly impaired hypoxia-induced luciferase activity (Figure 3-6).

![Figure 3-6](image)

**Figure 3-6. This site of SLC1A3 was a functional HRE.** Hep3B cells were co-transfected with pGL2-promoter-SLC1A3 HRE-WT, or SLC1A3 HRE-MUT (5’-ACGTG-3’ to 5’AAAAG-3’), and pSV-Renilla luciferase, then exposed to 20% O\textsubscript{2} or 1% O\textsubscript{2} for 24 h. The ratio of Firefly/Renilla was calculated and normalized to SLC1A3 HRE-WT at 20% O\textsubscript{2}. ***p < 0.001 vs. 20% O\textsubscript{2} WT, ###p < 0.001 vs. 1% O\textsubscript{2} WT. Bonferroni post test after ANOVA (mean ± SEM; n = 4).
Taken together, the results indicate that HIF-dependent expression of SLC1A1 and SLC1A3 leads to increased transport of glutamate out of Hep3B cells under hypoxic conditions.

**Hypoxia stimulates Hep3B cell proliferation**

When measuring the extracellular glutamate level in Hep3B cells exposed to hypoxia, we normalized the concentration to cell number. In doing so, we found that the proliferation of Hep3B cells was significantly increased under hypoxic conditions (Figure 3-7).

![Graph showing cell proliferation under different oxygen conditions](image)

**Figure 3-7. Hypoxia stimulates Hep3B cells proliferation.** Hep3B cells were exposed to 20% O₂ or 1% O₂ for indicated time and the cell numbers were counted. Hypoxia treatment was started 24h after seeding the cells as indicated by the arrow. **p <0.01, ***p <0.001 vs. 20% O₂. Bonferroni post test after ANOVA (mean ± SEM; n = 3).
**Glutamate receptor signaling stimulates proliferation of hypoxic Hep3B**

This observation suggested that under hypoxic conditions, Hep3B cells increase their expression of glutamate transporters SLC1A1 and SLC1A3, which facilitate increased release of glutamate, which then binds to glutamate receptors and triggers downstream signaling pathways leading to increased proliferation. Since the mitogen-activated protein kinase (MAPK) signaling cascade leading to the phosphorylation of ERK1/2 is a major pathway for extracellular signal-induced cell proliferation, we examined ERK1/2 phosphorylation in cells exposed to vehicle or antagonists of various classes of glutamate receptors, including the NMDA receptor antagonist MK-801 and AMPA receptor antagonist GYKI 52466. ERK1/2 phosphorylation was increased in vehicle-treated cells exposed to 1% O₂. Hypoxia-induced ERK1/2 phosphorylation was inhibited by GYKI 52466, but not by MK-801. ERK1/2 is activated by the upstream MAPK/ERK kinase (MEK) and U0126, an inhibitor of MEK, also impaired ERK1/2 phosphorylation (Figure 3-8).

**Figure 3-8. AMPA receptor antagonist inhibits the hypoxia increased ERK phosphorylation.** Cells were exposed to 20% or 1% O₂ + vehicle, 20 μM U0126, 250 μM MK-801, or 250 μM GYKI 52466 for 24 h. Whole cell...
lysate were analyzed by immunoblotting assays. Phosphorylated ERK (pERK) and total ERK (tERK) were evaluated.

Incubation of Hep3B cells with GYKI 52466 resulted in a significant inhibition of proliferation under 1% O₂ (Figure 3-9A), whereas U0126 inhibited cell proliferation under both 20% O₂ and 1% O₂ (Figure 3-9B).

![Graphs A, B, C showing cell number under different conditions](image)

**Figure 3-9. AMPA receptor inhibits Hep3B cells proliferation.** (A-C) Cells were exposed to 20% or 1% O₂ + vehicle or 250 μM GYKI 52466 (A) or + vehicle or 20 μM U0126 (B) or + vehicle or 250 μM MK-801 (C) for 96 h and counted. *p<0.05 vs vehicle-20%, ##p<0.01 vs vehicle-1%, Bonferroni post test after ANOVA (mean ± SEM; n = 3).

Although MK-801 had no effect on hypoxia-induced ERK1/2 phosphorylation, it did reduce cell proliferation (Figure 3-9C), which is consistent with a previous report that MK-801 blocks growth of hepatocellular carcinoma cells by inhibiting FOXO activity (Yamaguchi et al., 2013). Taken together, these results indicate that glutamate released by hypoxic Hep3B cells binds to AMPA receptors and stimulates MEK-ERK signaling, leading to increased proliferation.
**HIF-1 mediates hypoxia-induced expression of glutamate receptors**

AMPA receptors are homotetramers or heterotetramers composed of subunits GluR1 to GluR4, which are encoded by *GRIA1* to *GRIA4*. AMPA receptors are responsible for the vast majority of fast excitatory synaptic transmission within the mammalian central nervous system (Traynelis et al., 2010). Gene expression of AMPA receptors subunits *GRIA1* to *GRIA4* were analyzed (Figure 3-10A). *GRIA2* and *GRIA3* mRNA was induced by hypoxia in Hep3B cells but not in two breast cancer cell lines (Figure 3-10B-C), indicating that hypoxia-induced AMPA receptor expression was also cell-type specific.

**Figure 3-10.** *GRIA2* and *GRIA3* gene expression were dependent on HIFs. (A) Hep3B cells were exposed to 20% O$_2$ or 1% O$_2$ for 24h. AMPA receptor genes *GRIA1*, *GRIA2*, *GRIA3* and *GRIA4* mRNA were analyzed by real-time PCR and ratios were normalized to results obtained at 20% O$_2$. N.A. for not amplified after 40 cycles of amplification. *p <0.05, **p <0.01 vs. 20% O$_2$. Student’s $t$-test (mean ± SEM; n = 3). (B-C) MDA-MB-231 and MDA-MB-435 cells were exposed to 20% or 1% O$_2$ for 24 h. *GRIA2* (B)
and GRIA3 (C) mRNAs were analyzed by RT-qPCR and normalized to 20% O$_2$. (mean ± SEM; n = 3).

Hypoxic induction of GRIA2 and GRIA3 mRNA expression in Hep3B cells was abrogated when HIF-1α or both HIF-1α and HIF-2α were knocked down (Figure 3-11A-B).

**Figure 3-11.** GRIA2 and GRIA3 gene expression were dependent on HIF. (A-B)

GRIA2 (A) and GRIA3 (B) mRNA was analyzed in Hep3B subclones exposed to 20% or 1% O$_2$ for 24 h. *p<0.05 vs shNT-20%; #p<0.05 vs shNT-1%, two-way ANOVA/Bonferroni post-test. (mean ± SEM; n = 3).

**GRIA2 is a direct HIF-1α target gene**

ChIP assays revealed that a DNA sequence encompassing two copies of 5’-GCGTG-3’, with one copy at -26 nt and the other copy at +15 nt relative to the GRIA2
transcription start site (Figure 3-12A) was enriched by immunoprecipitation of chromatin from hypoxic Hep3B cells with HIF-1α or HIF-1β antibodies (Figure 3-12B).

![Schematic view of the GRIA2 gene](image)

**Figure 3-12.** A candidate site compassing HRE of *GRIA2* was enriched. (A)

Schematic view of the *GRIA2* gene. Exons were indicated by the black bars and the candidate HRE was indicated by the grey oval. (B) Hep3B cells were exposed to 20% O₂ or 1% O₂ for 24 h. ChIP assay was performed using IgG, HIF-1α, HIF-2α and HIF-1β antibodies. Specific primers flanking *GRIA2* HRE were used for qPCR. *p < 0.05, **p < 0.01 vs. the corresponding antibody at 20% O₂. Bonferroni post test after ANOVA (mean ± SEM; n = 3).

A 55-bp wild-type sequence spanning the HIF binding sites (WT), or the same sequence with mutation of 5'-GCGTG-3' to 5'-GAAAG-3' (MUT), was inserted into
pGL2-promoter. Hep3B cells were co-transfected with GRIA2 HRE-WT or HRE-MUT reporter together with pSV-Renilla, and exposed to 20% or 1% O\textsubscript{2} for 24 h. The ratio of firefly:Renilla luciferase increased with hypoxic exposure of cells transfected with GRIA2 HRE-WT whereas hypoxia-induced luciferase activity was impaired in cells transfected with HRE-MUT (Figure 3-13).

Figure 3-13. This site of GRIA2 was a functional HRE. Hep3B cells were co-transfected with pGL2-promoter-GRIA2 HRE-WT, or GRIA2 HRE-MUT (5’-GCGTG-3’ to 5’GAAAG-3’), and pSV-Renilla luciferase, then exposed to 20% O\textsubscript{2} or 1% O\textsubscript{2} for 24 h. The ratio of Firefly/Renilla was calculated and normalized to GRIA2 HRE-WT at 20% O\textsubscript{2}. ***p < 0.001 vs. 20% O\textsubscript{2} WT, ###p < 0.001 vs. 1% O\textsubscript{2} WT. Bonferroni post test after ANOVA (mean ± SEM; n = 4).

Taken together, these results indicate that GRIA2 and GRIA3 expression is induced by hypoxia in a HIF-dependent manner and that, at least in the case of GRIA2, this results from direct binding of HIF-1 to the target gene.
**FYN functions as a HIF-regulated intermediary between GRIA2/3 and ERK1/2**

It has been reported that the Src family tyrosine kinase LYN physically associated with AMPA receptors and functionally signaled to MAPK pathways in primary cell cultures from the cerebellum (Hayashi et al., 1999), whereas additional SRC family kinases expression were regulated by HIFs in ccRCC cell lines (Razorenova et al., 2011). We tested several SRC family kinases including SRC, LYN, LCK and FYN under hypoxia, and observed that in Hep3B cells, SRC, LYN and LCK expression were not affected under 1% O₂ (Figure 3-14).

![Image of immunoblot assays](image)

**Figure 3-14. Expression of SRC family kinases in hypoxic Hep3B cells.** Hep3B cells were exposed to 20% or 1% O₂ for 24 h. Immunoblot assays were performed using indicated Src family kinases antibodies.

We did observe that FYN expression was induced under 1% O₂. We further investigated whether *FYN* was regulated by HIFs in Hep3B cells and whether it transduced signals from AMPA receptors to ERK. FYN mRNA (Figure 3-15A) and
protein (Figure 3-15B) levels were induced by hypoxia in the shNT subclone and this induction was abrogated in the sh1α and sh1α/2α subclones, but not in the sh2α subclone.

Figure 3-15. *FYN* gene expression was dependent on HIF. (A) *FYN* mRNA was analyzed by real-time PCR in Hep3B subclones exposed to 20% O₂ or 1% O₂ for 24 hours. *p < 0.05 vs. shNT at 20% O₂, #p < 0.05 vs. shNT 1% O₂. Two-way ANOVA with Bonferroni post test. (mean ± SEM; n = 3). (B) Hep3B subclones were cultured at 20% O₂ or 1% O₂ for 48 h. *FYN* protein expression was determined by immunoblot assay. β-actin was used as loading control.

*FYN* mRNA expression was not induced by hypoxia in two breast cancer cell lines (Figure 3-16).
**Figure 3-16.** *FYN* gene expression was not affected by hypoxia in breast cancer cells. MDA-MB-231 and MDA-MB-435 cells were exposed to 20% or 1% O2 for 24 h. *FYN* mRNAs were analyzed by RT-qPCR and normalized to 20% O2. (mean ± SEM; n = 3).

We tried to identify HIF binding sites in *FYN* using the conventional method by scanning the gene sequence of *FYN* for 5’-RCGTG-3’, and picking some sites to design primers for real-time qPCR after chromatin immunoprecipitation. The *FYN* gene is 213 kb long including more than 200 sites of 5’-RCGTG-3’ and unfortunately we didn’t enrich any of the sites we picked including one site about 540 bp upstream of the transcription start site containing 5’-ACGTG-3’ followed by a 5’-CACA-3’ sequence which is found in many HREs (Fukuda et al., 2007) and one site about 200 bp upstream of the transcription start site containing two copies of 5’-GCGTG-3’ which are separated by 68 bp.

So we sought to find a way to limit the 5’-RCGTG-3’ candidates. The genome-wide identification of HIF binding sites were reported by coupling HIF-1α and HIF-2α chromatin immunoprecipitation to microarrays (ChIP-chip) (Krieg et al., 2010; Mole et al., 2009; Xia and Kung, 2009; Xia et al., 2009) or high-throughput sequencing (ChIP-Seq) (Schodel et al., 2011). Some of these data are not available while the rest didn’t provide much insight when trying to locate HIF binding sites at a specific gene as tested by Semenza lab members. Possible explanations include cell-specific response to hypoxia which is largely determined by DNA accessibility to the transcription factors (Bell et al., 2011). The high-resolution ChIP-Seq data combining the ENCODE DNase I
hypersensitivity data obtained in normoxic cells does provide evidence that actual HIF binding is correlated with DNA accessibility (Schodel et al., 2011).

In light of this, we examined the integrated transcription regulation from ENCODE tracks of the FYN gene. Monomethylated histone H3 lysine 4 (H3K4Me1) is often found near regulatory elements and histone H3 acetylated at lysine 27 (H3K27Ac) is often found near active regulatory elements. Both tracks represent layered data from 7 cell lines from ENCODE. The overlayed H3K4Me1 and H3K27Ac tracks are suggestive of enhancer or other regulatory activity. Yet these histone modifications are broad and the actual enhancers or other regulatory elements are typically just a small portion of the area marked by these modifications. However, regulatory regions are usually accessible and thus tend to be DNase sensitive in general. The ENCODE consortium provides the DNase Clusters track, which shows regions where the chromatin is hypersensitive to DNase I assayed in 125 cell types. We first identified overlapping H3K4Me1 and H3K27Ac peaks. Then those DNase I hypersensitivity clusters that appeared in more than 60 different cell lines out of 125 within these peaks were picked. DNA sequences of these clusters were retrieved and searched for the HIF binding site consensus sequence 5’-RCGTG-3’. We designed primers for 4 of these regions containing 5’-RCGTG-3’ for real-time qPCR after chromatin immunoprecipitation (Figure 3-17).
Figure 3-17. **UCSC genome browser display of transcriptional regulatory tracks of***

**FYN.** H3K4Me1, H3K27Ac peaks and DNase clusters at the FYN gene locus. Four 5’-RCGTG-3’ motifs within overlapping H3K4Me1 and H3K27Ac peaks and DNase I hypersensitivity clusters that appeared in more than 60 different cell lines are shown. Each different color in the H3K4Me1, H3K27Ac peaks represent one cell line shown in the right corner.

Significant hypoxia-induced binding of HIF-1α and HIF-1β to two of these four sites were seen (the leftmost two RCGTG motifs in Figure 3-17). These two HIF-1 binding sites, which contained the sequence 5’-ACGTG-3’, resided in intron 2 and intron 13 of the FYN gene (designated HRE#1 and HRE#2, respectively) (Figure 3-18).
Figure 3-18. *FYN* was enriched under hypoxia in ChIP assays. (A-B) Hep3B cells were exposed to 20% O₂ or 1% O₂ for 24 h. *FYN* HRE#1 was enriched by ChIP assay using IgG, HIF-1α, HIF-2α (A) and HIF-1β (B) antibodies. (C-D) Hep3B cells were exposed to 20% O₂ or 1% O₂ for 24 h. *FYN* HRE#2 was enriched by ChIP assay using IgG, HIF-1α, HIF-2α (C) and HIF-1β (D) antibodies. *p < 0.05, **p < 0.01 vs. the corresponding antibody at 20% O₂. Bonferroni post test after ANOVA (mean ± SEM; n = 3).

A 55-bp sequence spanning either wild type (WT) or mutant (MUT; 5’-ACGTG-3’ to 5’-AAAAG-3’) HIF binding site was inserted into pGL2-promoter. Firefly luciferase activity was significantly increased by hypoxia in Hep3B cells transfected with *FYN*
HRE#1-WT (Figure 3-19A) or FYN HRE#2-WT (Figure 3-19B). Compared to the respective WT construct, the hypoxic induction was significantly decreased in cells transfected with HRE#1-MUT or HRE#2-MUT.

Figure 3-19. These sites of FYN were functional HREs. (A-B) Hep3B cells were co-transfected with pGL2-promoter-FYN HRE#1-WT, or FYN HRE#1-MUT (5’-GCGTG-3’ to 5’GAAAG-3’) (A), or pGL2-promoter-FYN HRE#2-WT, or FYN HRE#2-MUT (5’-GCGTG-3’ to 5’GAAAG-3’) (B), and pSV-Renilla luciferase, then exposed to 20% O$_2$ or 1% O$_2$ for 24 h. The ratio of Firefly/Renilla was calculated and normalized to HRE-WT at 20% O$_2$. ***p < 0.001 vs. 20% O$_2$ WT, ###p < 0.001 vs. 1% O$_2$ WT.

Bonferroni post test after ANOVA (mean ± SEM; n = 4).

To confirm that FYN signals to ERK1/2, we stably knocked down FYN expression in Hep3B cells with two different shRNAs, shFYN-1 and shFYN-2 (Figure 3-20).
Figure 3-20. *FYN* knockdown in Hep3B cells. Hep3B subclones with FYN knockdown were exposed to 20% or 1% O$_2$ for 24 h. mRNAs were analyzed by RT-qPCR and normalized to shNT-20% O$_2$. **p<0.01, ***p<0.001 vs 20%-shNT, ###p<0.001 vs 1%-shNT, ANOVA/Bonferroni post-test. (mean ± SEM; n = 3).

ERK phosphorylation was induced by hypoxia in the shNT subclone, but not in the shFYN-1 or shFYN-2 subclone (Figure 3-21A). In addition, hypoxia significantly increased proliferation of the shNT subclone but not the shFYN subclones (Figure 3-21B).

Figure 3-21. *FYN* knockdown inhibits Hep3B cells proliferation. (A) Immunoblot assays were conducted using lysates prepared from Hep3B control (shNT)
and two FYN knockdown (shFYN-1 and shFYN-2) cells exposed to 20% O₂ or 1% O₂ for 24 h. (B) Cells were incubated in 20% or 1% O₂ for 72 h and counted. *p<0.05 vs shNT-20%, ##p<0.01, ###p<0.001 vs shNT-1%, ANOVA/Bonferroni post-test. (mean ± SEM; n = 3).

Taken together, these results indicate that FYN is a HIF-regulated intermediary that is required for the activation of ERK1/2 signaling and proliferation of Hep3B cells exposed to hypoxia.

**AMPA receptor antagonist inhibits Hep3B tumor xenograft growth**

Having demonstrated that glutamate → AMPA-type glutamate receptor → FYN → ERK signaling stimulates the proliferation of hypoxic Hep3Bs, we next asked whether inhibiting this pathway using the AMPA receptor antagonist, GYKI 52466, would have anti-cancer effects in vivo.

Hep3B cells were injected subcutaneously into the right flank of severe combined immune deficiency (SCID) mice. Tumor xenografts were allowed to grow to ~200 mm³ and then the mice were randomly divided into two groups that received daily intraperitoneal injections of either vehicle or GYKI 52466 (5 mg/kg). Administration of the AMPA receptor antagonist significantly impaired tumor growth (Figure 3-22A) with no effect on body weight (Figure 3-22B).
Figure 3-22. AMPA receptor antagonist inhibits Hep3B tumor xenografts growth.

(A-B) Hep3B cells were mixed with Matrigel and injected subcutaneously into SCID mice. When xenografts reached 200 mm$^3$, the mice were treated with vehicle or GYKI 52466. Tumor volumes (A) and body weight (B) were monitored. The arrow indicates the starting point of treatment. *p <0.05 vs vehicle, ANOVA/Bonferroni post-test (mean ± SEM; n = 3).

Tumors were harvested on day 45 and immunoblot assays of tumor lysates were performed to analyze levels of phosphorylated and total ERK1/2. Tumors from GYKI 52466 treated mice had a significantly decreased ratio of phosphorylated:total ERK (Figure 3-23).
Figure 3-23. AMPA receptor antagonist inhibits ERK phosphorylation in tumor xenografts. (A-B) Tumor lysates were subjected to immunoblot assays (A) and the ratio of phosphorylated:total ERK (pERK/tERK) was determined (B). *p<0.05 vs vehicle, Student’s t-test (mean ± SEM; n = 3).

Tumor sections analyzed by Ki67 immunohistochemistry, as a measure of cancer cell proliferation in vivo, revealed a significant decrease in the number of Ki67+ cells in xenografts from mice treated with GYKI 52466 (Figure 3-24).

Figure 3-24. AMPA receptor antagonist decreases Ki67+ cells in tumor xenografts. (A-B) Ki67 immunohistochemistry was performed with the tumor section
(A) and the Ki67 positive cells was quantified by image analysis (B).

*p<0.05 vs vehicle, Student’s t-test (mean ± SEM; n = 3).

**SLC1A1/3, GRIA2/3, and FYN gene expression in hepatocellular carcinoma patients**

To determine the clinical relevance of the glutamate signaling pathway in hepatocellular carcinoma, PROGgene, a gene expression based survival analysis web application for multiple cancers, was used to assess survival data. Of the two available liver cancer dataset at PROGgene, GSE10141 (Hoshida et al., 2008) was analyzed since it contained all 5 genes: *SLC1A1, SLC1A3, GRIA2, GRIA3* and *FYN*. (Figure 3-25).
Figure 3-25. Survival analysis for Hepatocellular carcinoma patients according to glutamate signaling pathway gene expression. Survival analysis performed on 80 Japanese hepatocellular carcinoma patients stratified by the average expression of multiple genes (SLC1A1, SLC1A3, GRIA2, GRIA3 and FYN).

Taken together, the data in Fig. 1-4 indicate that HIF-dependent SLC1A1/3 $\rightarrow$ GRIA2/3 $\rightarrow$ FYN $\rightarrow$ ERK1/2 signaling contributes to cell proliferation in Hep3B cells cultured under hypoxic conditions and in Hep3B tumor xenografts (Figure 3-26).
Figure 3-26. Schematic view of HIF mediated glutamate signaling pathway in Hep3B cells. Hypoxia leads to HIF1-α/HIF2-α accumulation and HIF1-α/HIF2-α transcribes multiple target genes in the glutamate signaling pathway: Glutamate transporters (SLC1A1 and SLC1A3) transports glutamate out of the cells which in turn binds to cell surface AMPA receptors (of which GRIA2 and GRIA3 are HIF target genes) and initiates cellular signaling pathways with FYN as an additional HIF target and signals downstream to ERK. The outcome is to promote Hep3B cells proliferation under hypoxic conditions.
Chapter 4

HIF-dependent glutamate signaling in clear cell Renal Cell Carcinoma (ccRCC) cells

*SLC1A1/3, GRIA3, and FYN expression correlate with HIF target genes in ccRCC*

Next, we investigated whether the glutamate → AMPA receptor → SRC-family-kinase signaling axis is active in other cancer cell types. Given the key role of HIFs in this pathway, ccRCC was analyzed, since VHL loss-of-function is common and represents an O₂-independent mechanism to activate HIFs in cancer cells. Analyzing gene microarray expression data from 42 ccRCCs in the TCGA database revealed that *SLC1A1*, *GRIA3* and *FYN* mRNA levels were each significantly correlated with the expression of six known HIF target genes (Table 4-1). *SLC1A3* mRNA levels were significantly correlated with 3 of the 6 target genes. For comparison, expression of *PKM2*, which is
another known HIF target gene, was also correlated with 3 of the 6 genes. RPL4 was analyzed as a representative non-HIF target gene. As expected, none of the above genes showed significant correlation with RPL4.

Table 4-1. Expression of SLC1A1, SLC1A3, GRIA3 and FYN is correlated with known HIF target genes expression in clear cell renal cell carcinomas.

<table>
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<td></td>
<td>VEGFA</td>
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<td>n.s.</td>
<td>n.s.</td>
<td>n.s.</td>
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</tbody>
</table>

*p <0.05, **p <0.01, ***p <0.001, n.s., not significant, Pearson’s correlation test of microarray data from The Cancer Genome Atlas (TCGA) dataset of 42 renal clear cell carcinomas.

39 of these 42 patients have survival data and we performed the survival analysis according to the median expression of gene examined (Figure 4-1). High SLC1A3 and GRIA3 expression have a tendency to correlate with worse survival, which is similar to the extent of SLC2A1, whereas high SLC1A1 and FYN expression have no effect on
patient survival, which is similar to the extent of VEGFA. The lack of significance might due to the small patient number in this dataset.

**Figure 4-1. ccRCC patient survival analysis according to individual gene expression.**

Survival analysis performed on 42 renal clear cell carcinomas from The Cancer Genome Atlas (TCGA) dataset. Patients were divided into two groups according to the median gene expression.

PROGgene was used to create prognostic plot for multiple genes (SLC1A1, SLC1A3, GRIA2, GRIA3 and FYN) in clear cell Renal Cell Carcinoma patients. GSE29609 was analyzed (Figure 4-2). There is a clear stratification of survival status according to the gene expression of the glutamate signaling pathway. However, no statistical significance was reached. Again, the small size of the cohort (39 tumors) could explain the lack of significance.
Figure 4-2. Survival analysis for clear cell Renal Cell Carcinoma patients according to the glutamate signaling pathway gene expression. Survival analysis performed on 39 frozen tissue from recent nephrectomies stratified by the average expression of multiple genes (\textit{SLC1A1, SLC1A3, GRIA2, GRIA3} and \textit{FYN}).

These data indicate that HIF-regulated glutamate receptor signaling may be clinically relevant in ccRCC.

\textit{HIF-dependent glutamate signaling stimulates survival of 786-O cells}

Among ccRCC with VHL loss-of-function, one-third of tumors overexpress exclusively HIF-2α (“H2” subtype) and two-thirds overexpress both HIF-1α and HIF-2α (“H1H2” subtype) (Gordan et al., 2008). Two \textit{VHL} mutant ccRCC cell lines, 786-O and
RCC4, which represent the H2 and H1H2 subtypes, respectively, were chosen for further analysis.

Only HIF-2α was constitutively expressed in 786-O cells and, after reintroduction of an expression vector encoding wild-type VHL, HIF-2α expression was markedly decreased under non-hypoxic conditions (Figure 4-3A). A decrease in SLC1A1, SLC1A3, GRIA3 and FYN protein and mRNA levels occurred in 786-O-VHL when HIF-2α overexpression was abrogated (Figure 4-3A-B).

![Image](image.png)

**Figure 4-3. HIF-mediated gene expression involved in glutamate signaling in 786-O ccRCC cells.** (A-B) Immunoblot (A) and RT-qPCR (B) assays of 786-O (-) and 786-O-VHL (+) cells. **p<0.01, ***p<0.001 vs 786-O.

In contrast, SLC1A2 and SLC7A11 mRNA levels were not affected by VHL loss-of-function (Figure 4-4).
**Figure 4-4.** *SLC1A2* and *SLC7A11* mRNA levels were not affected by VHL loss-of-function in 786-O cells. mRNAs were analyzed by RT-qPCR and normalized to 786-O.

Extracellular glutamate accumulated in a time-dependent manner in 786-O and 786-O-VHL cells cultured for 24, 48, or 72 h. However, conditioned medium from 786-O cells contained significantly more glutamate than 786-O-VHL at every time point (Figure 4-5).

**Figure 4-5.** Increased extracellular glutamate in the media of 786-O cells. Glutamate levels in media were measured, corrected for cell number, and normalized to results for 786-O cells cultured for 24 h. ***p<0.001 vs 786-O-48 h, **p<0.01 vs 786-O-72 h, Student’s t-test.
To elucidate any potential contribution of vesicular glutamate transporters or the cystine-glutamate antiporter, we examined the effects of inhibitors of these transporters, Evans Blue and Sulfasalazine (Sharma et al., 2010), respectively, on extracellular glutamate levels. Neither inhibitor affected cell proliferation (Figure 4-6A-B) or extracellular glutamate concentrations (Figure 4-6C-D). These results suggest that glutamate efflux in 786-O cells is mediated by the membrane glutamate transporters SLC1A1 and SLC1A3.
Figure 4-6. Vesicular glutamate transporters and cystine-glutamate antiporter do not contribute to elevated glutamate efflux by 786-O cells. (A-B) Cells were treated for 48 h with vehicle (Veh), or with indicated concentration of Sulfasalazine (A) or Evans Blue (B), and counted. (C-D) Glutamate levels in media treated with indicated concentration of Sulfasalazine (C) or Evans Blue (D), were measured, corrected for cell number, and normalized to results for 786-O treated with Veh. Data are mean ± SEM from ≥ 3 experiments.

In contrast to the effect of hypoxia on Hep3B cells, 786-O cells did not have any growth advantage over 786-O-VHL cells. However, 786-O cells exhibited decreased apoptosis compared to 786-O-VHL cells, as measured by Annexin V staining using flow cytometry (Figure 4-7).
Figure 4-7. Analysis of apoptosis in 786-O and 786-O-VHL cells. Spontaneous apoptosis in 786-O and 786-O-VHL cells was measured by PE-Annexin V staining by flow cytometry (upper panel). Q3, representing early apoptosis, was quantified and normalized to 786-O (lower panel). Data are mean ± SEM from ≥ 3 experiments.

The PI3K/AKT pathway is a major anti-apoptotic signaling pathway and AKT phosphorylation was increased, and cleaved (activated) caspase 3 was decreased, in 786-O as compared to 786-O-VHL cells (Figure 4-8).
Figure 4-8. Analysis of proteins involved in apoptosis in 786-O and 786-O-VHL cells.

Immunoblot assays were performed to detect: phosphorylated AKT (pAKT) and total AKT (tAKT); and full length and cleaved Caspase-3.

To determine whether glutamate → glutamate receptor → SRC family kinase signaling was involved, we treated 786-O cells with either the AMPA receptor antagonist GYKI 52466 or the SRC family kinase inhibitor Saracatinib. Both inhibitors decreased AKT phosphorylation and increased cleaved caspase-3 levels (Figure 4-9).
Figure 4-9. AMPA antagonist and SRC family kinase inhibitor increase apoptosis in 786-O cells. (A-B) 786-O cells were treated with vehicle, 500 μM GYKI 52466 (A) or 10 μM Saracatinib (B) for 24 h and cell lysates were subjected to immunoblot assays for phosphorylated AKT (pAKT), total AKT (tAKT), full length and cleaved Caspase-3.

Taken together these data indicate that in 786-O ccRCC cells, HIF-2 → SLC1A1/3 → GRIA3 → FYN → AKT signaling stimulates cell survival by inhibiting apoptosis (Figure 4-10).
Figure 4-10. The mechanisms and consequences of HIF-mediated glutamate signaling in 786-O cells. VHL mutation in ccRCC cells leads to HIF2-α accumulation in 786-O cells and HIF2-α transcribes multiple target genes in the glutamate signaling pathway: Glutamate transporters (SLC1A1 and SLC1A3) transports glutamate out of the cells which in turn binds to cell surface AMPA receptors (of which GRIA3 is a HIF2-α target gene in 786-O) and initiates cellular signaling pathways with FYN as an additional HIF target and signals downstream to AKT. The outcome is to increase cell anti-apoptosis ability in 786-O cells.

HIF-dependent glutamate signaling stimulates RCC4 migration and invasion

To further confirm the involvement of glutamate signaling in ccRCC cells, we analyzed RCC4 cells, which are VHL-null cells that constitutively express both HIF-1α and HIF-2α protein under non-hypoxic conditions. Stable transfection of an expression vector encoding wild-type VHL abolished the overexpression of HIF-1α and HIF-2α protein (Figure 4-11A). Similarly, expression of the glutamate transporters SLC1A1 and SLC1A3 were decreased at the protein (Figure 4-11A) and mRNA (Figure 4-11B) levels in the RCC4-VHL subclone. In contrast, SLC1A2 and SLC7A11 mRNA levels were not affected by VHL loss-of-function (Figure 4-11C).
Figure 4-11. Analysis of glutamate transporter and receptor gene expression in RCC4 and RCC4-VHL cells. (A) Immunoblot assays of RCC4 (-) and RCC4-VHL (+) cells were performed. (B-C) RT-qPCR assays of RCC4 and RCC4-VHL cells were performed *p<0.05, **p<0.01 vs RCC4, Student’s t-test. mRNAs were analyzed by RT-qPCR and normalized to RCC4. Data are mean ± SEM from ≥ 3 experiments.

Compared to 786-O cells, RCC4 cells responded differently to re-expression of wild type VHL. First, overexpression of glutamate receptor GRIA2 rather GRIA3 was extinguished (Figure 4-11A-B); second, expression of FYN was not affected, but the phosphorylation of LYN, another SRC family kinase member, was decreased in RCC4-VHL cells (Figure 4-11A). However, as in the case of 786-O, VHL-null RCC4 cells released significantly more glutamate than the RCC4-VHL subclone (Figure 4-12).
**Figure 4-12. Increased extracellular glutamate in the media of RCC4 cells.**

Glutamate levels in media were measured, corrected for cell number, and normalized to results for RCC4 cells cultured for 24 h. **p<0.01 vs RCC4-24 h, ***p<0.001 vs RCC4-48h, Student’s t-test.**

We next analyzed RCC4 subclones that were stably transfected with an empty vector (shEV) or vector encoding HIF-1α (sh1α) or HIF-2α (sh2α) shRNA, or vectors encoding both HIF-1α and HIF-2α shRNAs (sh1α/2α) to determine whether HIF-1α or HIF-2α was specifically required for *SLC1A1* and *SLC1A3* expression in RCC4 cells. *SLC1A1* and *SLC1A3* mRNA (Figure 4-13A-B) and protein (Figure 4-14) overexpression were abrogated when HIF-1α, HIF-2α, or both were knocked down in RCC4 cells.
**Figure 4-13.** SLC1A1 and SLC1A3 mRNA expression are HIF dependent in RCC4 cells. (A-B) RT-qPCR assays were performed for SLC1A1 (A) and SLC1A3 (B) with RCC4 subclones. *p<0.05, **p<0.01 vs shEV, ANOVA/Bonferroni post-test.

**Figure 4-14.** SLC1A1 and SLC1A3 protein expression are HIF dependent in RCC4 cells. Immunoblot assays were performed with indicated antibodies in RCC4 subclones.
In contrast to the increased proliferation of Hep3B cells under hypoxia and the decreased apoptosis of VHL-null 786-O cells, we found that VHL-null RCC4 cells exhibited increased migration as compared to RCC4-VHL cells (Figure 4-15).

Figure 4-15. Analysis of migration in RCC4 and RCC4-VHL cells. Cells were seeded in a Boyden chamber and migration through uncoated inserts in response to FBS was determined by crystal violet staining. **p<0.001 vs RCC4. Data are mean ± SEM from ≥ 3 experiments.

Migration was significantly impaired when RCC4 cells were treated with the glutamate receptor antagonist GYKI 52466, whereas the antagonist did not affect RCC4-VHL cells (Figure 4-16A-B). In contrast, Saracatinib dramatically decreased migration in RCC4 and RCC4-VHL cells (Figure 4-16C), indicating that the activity of SRC family kinase is required for basal as well as HIF-dependent migration.
Figure 4-16. AMPA antagonist and SRC family kinase inhibitor inhibit migration in RCC4 cells. (A) RCC4 and RCC4-VHL cells were seeded in a Boyden chamber in the presence of vehicle, 250 μM GYKI 52466 and migration through uncoated inserts in response to FBS was determined by crystal violet staining. (B-C) Membranes were cut from the Transwell and rinsed in 1 mL of 33% acetic acid. Absorbance was read at 570 nm. Results were quantified for cell treated with GYKI 52466 (B) and 10 μM Saracatinib (C).
*p<0.05, ***p<0.001 vs RCC4-vehicle, ###p<0.001 vs RCC4-VHL-vehicle. Data are mean ± SEM from ≥ 3 experiments.

Similar results were seen in the invasion assay in which a layer of Matrigel was coated on top of the transwell inserts and cells were allowed to invade through it. Invasion was significantly impaired when RCC4 cells were treated with the glutamate receptor antagonist GYKI 52466, whereas the antagonist did not affect RCC4-VHL cells, whereas Saracatinib dramatically decreased invasion in RCC4 and RCC4-VHL cells (Figure 4-17).
Figure 4-17. AMPA antagonist and SRC family kinase inhibitor inhibit invasion in RCC4 cells. (A) RCC4 and RCC4-VHL cells were seeded on Matrigel-coated inserts in the presence of vehicle, 250 μM GYKI 52466. Cells that invaded through the coated inserts were determined by crystal violet staining. (B-C) Membranes were cut from the Transwell and rinsed in 1 mL of 33% acetic acid. Absorbance was read at 570 nm. Results were quantified for cell treated with GYKI 52466 (B) and 10 μM Saracatinib (C).
Taken together, the data indicate that HIF-1/2 → SLC1A1/3 → GRIA2 → LYN signaling stimulates migration and invasion of VHL-null RCC4 cells (Figure 4-18).

**Figure 4-18.** The mechanisms and consequences of HIF-mediated glutamate signaling in RCC4 cells. VHL mutation in ccRCC cells leads to HIF1-α/HIF2-α accumulation in RCC4 cells and HIF1-α/HIF2-α transcribes multiple target genes in the glutamate signaling pathway: Glutamate transporters (SLC1A1 and SLC1A3) transports glutamate out of the cells.
which in turn binds to cell surface AMPA receptors (of which *GRIA2* is a HIF target gene in RCC4) and initiates cellular signaling pathways. The outcome is to enhance migration and invasion in RCC4 cells.
Chapter 5

Discussion

The results presented here delineate novel molecular mechanisms by which HIFs mediate enhanced glutamate receptor signaling in Hep3B, 786-O, and RCC4 cancer cells through the direct, coordinate transcriptional activation of multiple genes encoding glutamate transporters and glutamate receptors in response to HIF activity resulting either from hypoxia or VHL loss-of-function. In Hep3B and 786-O cells, HIFs also activated transcription of the\textit{FYN} gene, which served to amplify signaling downstream of the glutamate receptors. Despite the similar HIF-mediated transcriptional responses and activation of SRC family kinases, the ultimate biological consequences of glutamate signaling were cell-type specific: in Hep3B cells, FYN signaled to ERK1/2 to stimulate cell proliferation; in 786-O cells, FYN signaled to AKT to stimulate cell survival; and in RCC4 cells, LYN signaling stimulated cell migration and invasion.
Hypoxia induces cancer cell glutamate efflux mediated by SLC1A1 and SLC1A3

There are several mechanisms explaining glutamate release in the CNS including: 1) vesicular glutamate transporters (encoded by genes SLC17A6 (a.k.a.VGLUT2), SLC17A7 (a.k.a.VGLUT1) and SLC17A8 (a.k.a.VGLUT3)), which mediate glutamate release form presynaptic neurons; 2) antiporter system \text{xc}^- (encoded by genes SLC7A11 (encoding the light chain xCT and conferring the specificity) and SLC3A2 (encoding the heavy chain 4F2hc)), which mediate exchange of one extracellular cystine for one intracellular glutamate; 3) membrane glutamate transporters (encoded by genes SLC1A1 (a.k.a.EAAT3), SLC1A2 (a.k.a.EAAT2 or GLT1), SLC1A3 (a.k.a.EAAT1 or GLAST), SLC1A6 (a.k.a.EAAT4) and SLC1A7 (a.k.a.EAAT5)), which mediate reversed uptake of glutamate in severe brain ischemia.

Prior studies demonstrated that cancer cells release glutamate, but we have now demonstrated that increased glutamate efflux occurs in hepatocellular carcinoma cells in response to hypoxic conditions and in ccRCC cells due to VHL loss-of-function. In both cases, augmented glutamate efflux results from HIF-dependent SLC1A1 and SLC1A3 expression. The membrane glutamate transporters encoded by these genes conventionally mediate glutamate transport into cells under physiological conditions, but glutamate transport is reversible, with glutamate efflux occurring in ischemic tissue (Grewer et al., 2008). Over 50% of glutamate release by glioma cell lines was attributed to the \text{xc}^- cystine/glutamate antiporter, which has been implicated in other cancer cells that release glutamate (Lo et al., 2008; Sharma et al., 2010). However, the reported increase of glutamate and decrease of cystine in the media was not equimolar, with 2- to 7-fold more...
glutamate secreted (Timmerman et al., 2013), which suggests that even under non-hypoxic conditions additional mechanisms must contribute to glutamate release in those cancer cells. SLC1A2, another major membrane glutamate transporter in CNS, mediates up to 95% of glutamate uptake in the brain and is frequently silenced in gliomas due to DNA methylation (Tanaka et al., 1997; Zschocke et al., 2007). Neither SLC7A11 nor SLCA2 mRNA levels were affected by HIF activation in Hep3B cells or ccRCC cells, and inhibitors of cystine/glutamate antiporter or vesicular glutamate transporter activity had no effect on extracellular glutamate levels in 786-O cells, excluding their contribution to HIF-mediated glutamate release in these cells.

**Glutamate receptor function is cancer cell-type specific**

Several prior studies reported functional effects of AMPA receptors in cancer. GRIA1 and GRIA3 were shown to promote tumor progression in glioma (de Groot et al., 2008; Piao et al., 2009) and pancreatic cancer (Ripka et al., 2010). In contrast, GRIA4 is subject to DNA methylation (Bennett et al., 2009; Kostareli et al., 2013) and inhibition of GRIA4 expression increased cancer cell proliferation (Luksch et al., 2011). In the case of GRIA2, there are conflicting reports regarding its role in cancer biology. GRIA2 expression is upregulated in uterine leiomyoma and gastrointestinal neuroendocrine carcinoma compared to adjacent normal tissues (Arslan et al., 2005; Leja et al., 2009; Tsibris et al., 2003). In contrast, GRIA2 expression is lost in high-grade glioma and forced expression in glioma cells inhibits proliferation and induces apoptosis (Beretta et al., 2009). (Table 5-1)
Table 5-1. Implications of AMPA receptor genes in cancer.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer type</th>
<th>Mouse model/ Human patients</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRIA1</td>
<td>Glioma cells</td>
<td>Subcutaneous xenograft</td>
<td>Pro-tumoral</td>
<td>(de Groot et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Glioma cells</td>
<td>Intracranial xenograft</td>
<td>Pro-tumoral</td>
<td>(Piao et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Glioblastoma Derived Brain Tumor Initiating Cells (BTICs)</td>
<td>Strongly expressed in GBM BTICs</td>
<td></td>
<td>(Oh et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Glioblastoma cells</td>
<td>Subcortical &amp; Subcutaneous xenograft</td>
<td>Anti-tumoral Ca(2+)-impermeable</td>
<td>(Ishiuchi et al., 2002)</td>
</tr>
<tr>
<td></td>
<td>Glioblastoma cells</td>
<td>Subcutaneous xenograft</td>
<td>Pro-tumoral Ca(2+)-permeable</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Leiomyoma</td>
<td>Leiomyoma</td>
<td>Pro-tumoral??? Up-regulated in 5 of the 8 microarray analyzed</td>
<td>(Arslan et al., 2005)</td>
</tr>
<tr>
<td>GRIA2</td>
<td>Gastrointestinal neuroendocrine carcinomas</td>
<td>Anti-tumoral Ca(2+)-impermeable</td>
<td></td>
<td>(Ishiuchi et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Gastrointestinal neuroendocrine carcinomas</td>
<td>Pro-tumoral??? mRNA specifically expressed in neuroendocrine carcinomas</td>
<td></td>
<td>(Leja et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Glioma cells</td>
<td>Anti-tumoral</td>
<td></td>
<td>(Beretta et al., 2009)</td>
</tr>
<tr>
<td></td>
<td>Ovarian serous carcinomas</td>
<td>Ovarian cancer</td>
<td>Anti-tumoral in vitro 1. Upregulated in chemosensitive tumours 2. High expression correlates with longer survival</td>
<td>(Choi et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>Solitary Fibrous Tumour</td>
<td>Solitary Fibrous Tumour</td>
<td>Pro-tumoral??? Top up-regulated by NAB2/STAT6 fusions</td>
<td>(Mohajeri et al., 2013)</td>
</tr>
<tr>
<td>GRIA3</td>
<td>Pancreatic Cancer cells</td>
<td>Subcutaneous xenograft</td>
<td>Pro-tumoral</td>
<td>(Ripka et al., 2010)</td>
</tr>
</tbody>
</table>
Table 5-1. Implications of AMPA receptor genes in cancer. (Cont.)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Cancer type</th>
<th>Mouse model/ Human patients</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
</table>
| GRIA2 | Oropharyngeal squamous cell carcinomas | Oropharyngeal squamous cell carcinomas (OPSCC) | Anti-tumoral??  
1. HPV-related OPSCC has higher methylation  
2. Combined promoter methylation pattern including high GRIA4 methylation correlated with improved survival | (Kostareli et al., 2013) |
| GRIA3 | Oropharyngeal squamous cell carcinomas | Oropharyngeal squamous cell carcinomas (OPSCC) | Anti-tumoral??  
1. HPV-related OPSCC has higher methylation  
2. Combined promoter methylation pattern including high GRIA4 methylation correlated with improved survival | (Kostareli et al., 2013) |

In pancreatic ductal adenocarcinoma, AMPA receptor signaling to KRAS and MAPK promotes migration and invasion (Herner et al., 2011). However, the molecular basis for AMPA receptor expression was not determined. In the current study, we demonstrate that HIF-dependent GRIA2 and GRIA3 expression in hepatocellular carcinoma and ccRCC cell lines promotes tumor progression through cell-type-specific effects on proliferation, survival, migration and invasion. Analysis of gene expression data from human ccRCC indicates that the same transcriptional circuits are active in vivo, thereby establishing the clinical relevance of our findings.
The tumor microenvironment is a trigger for glutamate receptor signaling

In a mouse model of pancreatic neuroendocrine tumor and in selected human cancers, increased interstitial fluid pressure was reported to be a stimulus for increased expression of vesicular glutamate transporters, leading to activation of NMDA-type glutamate receptors and downstream MEK-MAPK and CaMK signaling leading to proliferation and invasion (Li and Hanahan, 2013). In contrast, we show that hypoxia is a microenvironmental stimulus that triggers HIF-dependent expression of membrane glutamate transporters as well as AMPA-type glutamate receptors in hepatocellular carcinoma cells.

Therapeutic implications

This study demonstrates that HIF-mediated glutamate efflux and signaling via AMPA-type glutamate receptors promotes multiple aspects of cancer progression in a cell-type-specific manner and that administration of an AMPA receptor antagonist significantly inhibits Hep3B tumor xenograft growth. The AMPA receptor antagonist talampanel was well tolerated but had no activity as a single agent in a Phase II clinical trial involving patients with recurrent glioma (Iwamoto et al., 2010). Previously studies have demonstrated that administration of the HIF-1 inhibitor digoxin also significantly inhibits Hep3B tumor xenograft growth (Zhang et al., 2008) and HIF inhibitor also impairs ccRCC tumor growth (Chintala et al., 2012). In addition to blocking glutamate receptor signaling, HIF inhibitors block many other pathways that are critical for cancer progression (Harris, 2002; Onnis et al., 2009; Semenza, 2003; Semenza, 2010a; Xiang et
al., 2013). Further studies are needed to evaluate the potential benefit of HIF inhibitors in the treatment of advanced liver and kidney cancers, which respond poorly to current chemotherapy regimens.
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Wellcome Trust Epigenomics of Common Diseases Conference, Baltimore, MD 2012
50th Annual Course on Medical and Experimental Mammalian Genetics
The Jackson Laboratory, Bar Harbor, ME 2009
Summer Workshop on Stem Cell, Development and Evolution
Kunming Institute of Zoology, Chinese Academy of Sciences, Kunming, China 2007