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Kristen M. Holmes

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By

Kristen M. Holmes, M.S.

APPROVED:

_____
Wei Zhang, Ph.D., Supervisory Professor

_____
Gregory N. Fuller, M.D., Ph.D.

_____
Elsa Flores, Ph.D.

_____
Renata Pasqualini, Ph.D.

_____
Dihua Yu, M.D., Ph.D.

APPROVED:

_____
George M. Stancel, Ph.D.
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

ELUCIDATING THE IGFBP2 SIGNALING PATHWAY IN GLIOMA DEVELOPMENT AND PROGRESSION

A

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Kristen M. Holmes
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Elucidating the IGFBP2 signaling pathway in glioma development and progression

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Kristen M. Holmes, M.S.

Supervisory Professor: Wei Zhang, Ph.D.

Diffuse gliomas are highly lethal central nervous system malignancies which, unfortunately, are the most common primary brain tumor and also the least responsive to the very few therapeutic modalities currently available to treat them. IGFBP2 is a newly recognized oncogene that is operative in multiple cancer types, including glioma, and shows promise for a targeted therapeutic approach. Elevated IGFBP2 expression is present in high-grade glioma and correlates with poor survival. We have previously demonstrated that IGFBP2 induces glioma development and progression in a spontaneous glioma mouse model, which highlighted its significance and potential for future therapy. However, we did not yet know the key physiological pathways associated with this newly characterized oncogene. We first evaluated human glioma genomics data harnessed from the publicly available Rembrandt source to identify major pathways associated with IGFBP2 expression. Integrin and ILK, among other cell migration and invasion-related pathways, were the most prominently associated. We confirmed that these pathways are regulated by IGFBP2 in glioma cells lines, and demonstrated that 1) IGFBP2 activates integrin α5β1, leading to the activation of key pathways important in glioma; 2) IGFBP2 mediates cell migration pathways through ILK; and 3) IGFBP2 activates NF-κB via an integrin α5 interaction. We then sought to determine whether this was a physiologically active signaling pathway *in vivo* by assessing its ability to induce glioma progression in the RCAS/tv-a spontaneous glioma

mouse model. We found that ILK is a key downstream mediator of IGFBP2 that is required for the induction of glioma progression. Most significantly, a genetic therapeutic approach revealed that perturbation of any point in the pathway thwarted tumor progression, providing strong evidence that targeting the key players could potentially produce a significant benefit for human glioma patients. The elucidation of this signaling pathway is a critical step, since efforts to create a small molecule drug targeting IGFBP2 have so far not been successful, but a number of inhibitors of the other pathway constituents, including ILK, integrin and NF-κB, have been developed.

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Abbreviations

CNS	central nervous system
WHO	World Health Organization
GBM	glioblastoma multiforme
NF1/2	neurofibromatosis 1/2
RB1	retinoblastoma 1
IDH1/2	isocitrate dehydrogenase 1/2
MGMT	*O*6-methylguanine-DNA methyltransferase
RTK	receptor tyrosine kinase
EGFR	epidermal growth factor receptor
PDGFR	platelet-derived growth factor receptor
PI(3)K	phosphatidylinositol 3-kinase
PTEN	phosphatase and tensin homolog
TCGA	The Cancer Genome Atlas
VEGF	vascular endothelial growth factor
GEMM	genetically engineered mouse model
GFAP	glial fibrillary acidic protein
RCAS	Replication-Competent Avian leukosis virus long terminal repeat with a Splice acceptor
SVZ	subventricular zone
ID	inhibitor of DNA-binding
IGFBP2	Insulin-like growth factor (IGF) binding protein 2
ECM	extracellular matrix
HBD	heparin binding domain
RGD	Arginine-Glycine-Aspartic acid
MMP	matrix metalloproteinase
MIIP	migration and invasion inhibitory protein
HSC	hematopoietic stem cells
FAK	focal adhesion kinase
GFR	growth factor receptor

EGF	epidermal growth factor
PIP2	phosphatidylinositol (4,5)-biosphosphate
PyVmT	polyoma virus middle T antigen
NSCLC	non-small cell lung cancer
ILK	integrin-linked kinase
ANK	ankyrin
NF-κB	Nuclear factor-kappa B
EMT	epithelial to mesenchymal transition
TGFβ	tumor growth factor beta
SIRE	Snail ILK responsive element
RHD	Rel homology domain
IκB	inhibitor of κ B
TNF	tumor necrosis factor
IKK	IκB kinase
SASP	senescence-associated secretory phenotype

CHAPTER 1: Introduction

Glioma

Introduction. Gliomas are glial cell derived tumors of the brain and spinal cord, and are characterized by poor patient prognosis. Gliomas account for 31% of all primary Central Nervous System (CNS) tumors and 80% of all malignant primary CNS tumors (1). Current treatment regimens yield meager benefit, as patients with high grade, invasive glioma typically do not survive longer than a year following diagnosis (2).

The CNS is composed of a variety of cell types, including neurons, glia, and vascular cells (3). Neurons are cells that transmit electrical signals throughout the body, allowing a superhighway of information to circulate within the CNS radiating out to the peripheral nervous system (PNS). Glial cells are neural helper cells responsible for a variety of functions depending on the glial subtype. Although it was previously accepted that glia outnumbered neuronal cells 10:1, recent research has revealed that there are nearly equal numbers of neuronal and non-neuronal cells (4). The relative number of cell types, however, differs in various regions of the brain, with the highest ratio of non-neuronal to neuronal cells residing in the cerebral cortex. This pattern correlates with the distribution of mass within the brain (4). Glial cells are comprised of four cell types: astrocytes, oligodendrocytes, ependymal cells, and microglia. Glia perform various functions including supporting, protecting, and supplying nutrients to neuronal cells. Astrocytes are the most numerous type of glia in the brain and respond to inflammation by proliferating and migrating to the site of injury. Astrocytes also connect neurons to the blood supply and protect neurons from glutamate-induced excitotoxicity by taking up excess transmitter from

the synapse (5). Oligodendrocytes perform the critical function of myelinating neuronal axons, forming an insulative layer that allows efficient propagation of electrical signals. Ependymal cells make and secrete cerebral spinal fluid and form the lining of ventricles. Finally, microglia are the major immune cell of the brain and are known as the resident macrophage (3).

There are 4 main types of glioma: astrocytoma, oligodendroglioma, mixed (oligoastrocytoma), and ependymoma. The World Health Organization (WHO) has assigned a classification scheme that describes the tumors based on histopathological features, and separates the diffuse gliomas, including astrocytoma, oligodendroglioma, and oligoastrocytoma, into two major subtypes: 1) astrocytic tumors, including pilocytic astrocytoma, diffuse astrocytoma, and glioblastoma multiforme (GBM); and 2) oligodendroglial tumors, including oligodendroglioma and mixed oligoastrocytoma (6). Gliomas are further classified by WHO grade (I-IV): WHO Grade I is considered non-malignant and includes pilocytic astrocytoma; WHO Grade II is a low-grade malignant, well-differentiated tumor, including diffuse astrocytoma and oligodendroglioma; WHO Grade III is anaplastic, including anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma; WHO Grade IV includes GBM (6). Glioblastoma may be further subdivided into primary and secondary GBMs, with primary GBM forming *de novo*, with a short clinical history and no prior history of disease (includes >90% of GBM) (7), while secondary GBM is much less prevalent and develops from a previously diagnosed less malignant lesion (8). Astrocytoma and GBM comprise 76% of all gliomas (GBM alone comprises 53.7%), followed by oligodendroglioma and ependymoma, which account for 6.5% and 5.9% of all gliomas, respectively (1).

Gliomas occur in individuals of all ages; however, the tumor histological spectrum varies according to age: pilocytic astrocytoma is the most frequent glioma in children, whereas GBM is most common in adults (median age of 64 years), and rare in children (7). Younger patients (age < 45) are more likely to develop low-grade tumors. The incidence rate of GBM increases significantly around age 45 and continues this trend until age 84 (1). Gliomas are slightly more prevalent in men than women (incidence rate of 7.17 versus 5.08 per 100,000 person-years), and the incidence rate is significantly higher among blacks than whites (1).

Patient prognosis is highly dependent on glioma histology type and grade, in addition to patient age, mental status, postoperative Karnofsky or WHO performance scores (assess patient's overall condition), and extent of surgical resection (7). Oligodendroglial tumors have the most favorable prognosis, with one and five year survival rates of 94% and 79%, respectively. Patients with an anaplastic oligodendroglioma are typically faced with a 49% chance of surviving five years after diagnosis. Patients with either an astrocytoma or anaplastic astrocytoma are typically given 5-year survival rates of 48% or 27%, respectively. Patients with GBM have a 34.6% chance of surviving one year after diagnosis, and a meager 4.75% chance of surviving five years after diagnosis (1). Although low-grade diffuse gliomas typically yield a more favorable prognosis than their high-grade counterparts, these tumors have a strong propensity to progress to secondary GBM within 2-5 years (8).

The etiology of glioma is largely unknown (7, 9). Environmental agents have been examined, but ionizing radiation is the only agent that has been clearly associated with increased risk of glioma (9). Several studies have reported a familial link in glioma cases

(10-13), and it has been suggested that 5% of glioma cases are familial (11). Although infrequent, certain hereditary genetic syndromes predispose subjects to glioma development, including Li-Fraumeni (germline *TP53* mutation), neurofibromatosis 1 (*NF1* truncation or deletion), neurofibromatosis 2 (*NF2* germline mutations), and Turcot syndrome (*APC*, *MLH1*, *MSH2*, *PMS2* mutations) (9, 14). Additional susceptibility genes are retinoblastoma 1 (*RB1*) and tuberous sclerosis (*TSC1* and *TSC2*) (9). The increased risk of glioma among genetic syndromes underlines the genetic basis of glioma development.

Common molecular alterations in glioma. As observed in other cancers, gliomas form when multiple genes become altered or mutated. Specifically, tumor suppressor genes are lost or mutated while oncogenes are activated thus forming the underlying genetic landscape of glioma.

Codeletion of chromosome arms 1p19q is considered a genetic hallmark of oligodendroglioma (15). This genetic aberration is observed in 80-90% of low-grade oligodendroglioma and about 60% of anaplastic oligodendroglioma. In contrast, 1p19q codeletion is observed in <10% of astrocytic tumors, forming a genetic separation between oligodendroglial and astrocytic tumors (15, 16). Importantly, the classic morphology of oligodendroglioma, such as perinuclear halos and unique “chicken wire” vascular pattern is highly associated with 1p19q codeletion. It is noteworthy to mention that loss of the entire 1p and 19q is frequently observed. Partial loss of the chromosomes or deletion of 1p alone may carry a different prognosis than complete 1p19q codeletion (17, 18). It is well-documented that 1p19q predicts for increased survival rate and improved chemotherapy response (19-22), and it appears now that the 1p19q codeletion is more appropriately used as a predictive factor of response (and survival) to radio- or chemotherapies (23).

Recently, Parsons *et al.* (24) reported mutations in the isocitrate dehydrogenase 1 and 2 genes (*IDH1* and *IDH2*), which are involved in the citric acid cycle. *IDH1* and *IDH2* mutations have been reported as strong independent prognostic factors for survival (15). Subsequently, mutations in these genes have been identified in >68% of low-grade gliomas of both oligodendroglial and astrocytic lineages, in addition to secondary GBM (25-27). More than 90% of *IDH1* mutations occur in the active site and include R132H. *IDH1* mutations are now considered to be a critical initiating event in glioma. In fact, *IDH1* mutations have been reported to occur prior to *TP53* mutation or 1p19q codeletion in serial biopsies of the same patient tumor. (28). Another group found that all tumors harboring a complete 1p19q codeletion also contained *IDH1* or *IDH2* mutations (29). Therefore, it is suggested that *IDH* mutations may be a prerequisite for 1p19q codeletion and oligodendroglioma development (15).

In accordance with conventional tumor development, gliomas contain characteristic losses or mutations in key tumor suppressor genes, including *TP53*, *CDKN2A*, and phosphatase and tensin homolog (*PTEN*) (30). Gliomas are strongly impacted by mutation or loss of *TP53*, which encodes the “guardian of the genome,” p53. *TP53* mutations are considered to be an early event in glioma development and have been reported in >60% of diffuse astrocytomas, in ~40% of mixed oligoastrocytomas, and <5% in oligodendrogliomas (31, 32). Notably, *TP53* mutations and 1p19q codeletions are mutually exclusive in mixed oligoastrocytomas, indicating that these tumors are genetically monoclonal, resembling either oligodendroglioma or astrocytoma (31). *PTEN* is mutated or deleted in a large majority of GBMs, which directly affects phosphatidylinositol 3-kinase (PI(3)K) and Akt signaling (33). Homozygous deletion occurs in up to 36% of GBM (34), whereas loss of both copies rarely is observed in anaplastic and low-grade glioma (35, 36). Mutations are

also common in primary GBM, but rare in low-grade glioma (37). Promoter methylation, however, is observed in low-grade, anaplastic glioma, and secondary GBM and is associated with decreased PTEN protein levels (38); therefore, promoter methylation is also likely to play a role in low-grade glioma development. Further, *PTEN* expression levels and genetic losses are important prognostic factors for tumor grade and survival (33). The *CDKN2A* gene encodes 2 distinct proteins, p16INK4a, which prevents phosphorylation of RB1, thereby preventing G1/S cell cycle transition; and p14ARF, which binds to MDM2, thereby stabilizing p53 and preventing cell cycle progression. Similar to *PTEN*, *CDKN2A* genetic aberrations increase according to tumor grade. Homozygous deletion of *INK4a* and *ARF* is observed in 49% and 52% of GBM, respectively (34), whereas homozygous deletion of INK4a or ARF have been found in 25% of anaplastic oligodendroglioma, but absent in oligodendroglioma (39-41). Watanabe *et al.* (39) reported promoter hypermethylation of *p14ARF* in 21% of oligodendroglioma and in 15% of anaplastic oligodendroglioma; *p16INK4a* promoter hypermethylation was observed in only one anaplastic oligodendroglioma case. These data suggest a role of promoter hypermethylation in the transformation process and selection of homozygous deletion of *INK4a* and *ARF* in subsequent progression processes (39).

Receptor tyrosine kinases (RTK) are frequently overexpressed or mutated in glioma, and include epidermal growth factor receptor (EGFR), Platelet-derived growth factor receptor (PDGFR), ERBB2, and c-MET (30). Ligand binding results in activation of downstream Ras and PI(3)K signaling cascades that promote cell proliferation, invasion, and growth. Among these, EGFR is the most prevalent alteration, affecting ~45% of GBMs (34). Genomic amplification is the most frequently observed aberration (34) and usually co-occurs with point mutations and deletions in both the extracellular and cytoplasmic

domains. One such example is EGFR variant III (EGFRvIII), which lacks exons 2-5 of the extracellular domain, causing constitutive activation of the receptor (42, 43). PDGF signaling is altered in both low-grade glioma and 13% of GBM by amplification or mutation of the receptor, or overexpression of PDGF ligands (34, 44). Less frequently, mutation of *ERBB2* (part of the EGFR family) and amplification of *MET* are observed in 13% and 4% of GBMs, respectively (34).

The Cancer Genome Atlas (TCGA) research network launched a collaborative effort to investigate integrative large-scale molecular alterations in GBM by incorporating DNA copy number, DNA methylation, and gene expression (34). Overall, frequent genetic alterations occurred in three critical signaling pathways: 1) RTK signaling was altered in 88% (included amplification of *EGFR*, *PDGFRA*, *MET*, and *Akt*; mutations of *EGFR*, *ERBB2*, *NF1*, *RAS*, *PI3K*, *PTEN*, and *FOXO*; homozygous deletion of *PTEN* and *NF1*); 2) p53 signaling was altered in 87% (included amplification of *MDM2* and *MDM4*; homozygous deletion and mutation of *CDKN2A*(*ARF*) and *TP53*); and 3) RB signaling was altered in 78% (included homozygous deletion and mutation of *CDKN2A*(*INK4A*), *CDKN2B*, *CDKN2C*, *RB1*; amplification in *CDK4*, *CCND2*, *CDK6*). Subsequent work by Verhaak et al. (30) analyzed TCGA GBM genomics data and reported distinct subtypes of glioma based on molecular signatures: proneural, neural, classical, and mesenchymal. EGFR alterations defined the classical subype; NF1, the mesenchymal subtype; and PDGFRA/IDH1, the proneural subtype. They further demonstrated that these subtypes were important in response to aggressive therapies, with classical and mesenchymal groups having a statistically significant prolonged survival and while there was no difference in survival among the patients of the proneural group (45). Other investigators have also undertaken efforts to sub-classify GBM via transcriptional profiling, DNA sequence and copy number,

proteomic markers, and DNA methylation (46-52). These studies have been useful in identifying prognostically significant gene signatures and in sub-dividing GBM into distinct classes, reflecting the complexity of GBM (53).

Prognostic indicators are critical in order to optimally treat patients. One such important prognostic marker and molecular alteration in glioma is promoter methylation of *O*6-methylguanine-DNA methyltransferase (*MGMT*), which results in its epigenetic silencing (54). *MGMT* encodes a DNA repair enzyme that removes alkyl groups from the *O*6 position of DNA. The standard of care involves DNA alkylating agents such as nitrosourea or temozolomide, which impedes gene transcription and triggers apoptosis. Decreased levels of MGMT via promoter methylation would prevent tumor cells from repairing DNA alkylation and subsequently induce cell death. This is the most probable reason why *MGMT* promoter methylation is a strong response predictor to chemotherapies (55-57). Recent studies reported a favorable predictive response to radiotherapy alone (58), independent of either alkylating chemotherapy or radiotherapy (21, 59).

Histologic analysis remains the gold standard of glioma diagnosis (60). Features including cell density, microvascular proliferation, mitotic activity, necrosis, and nuclear atypia are utilized to distinguish glioma grade. However, morphological analyses are often subjective and do not distinguish the molecular alterations in the cell. For example, we now know that primary and secondary GBM have marked molecular differences (8); however, they are morphologically indistinguishable and are treated as a single disease. As researchers continue to uncover molecular pathogenesis of glioma, future diagnoses will most likely include molecular classifiers.

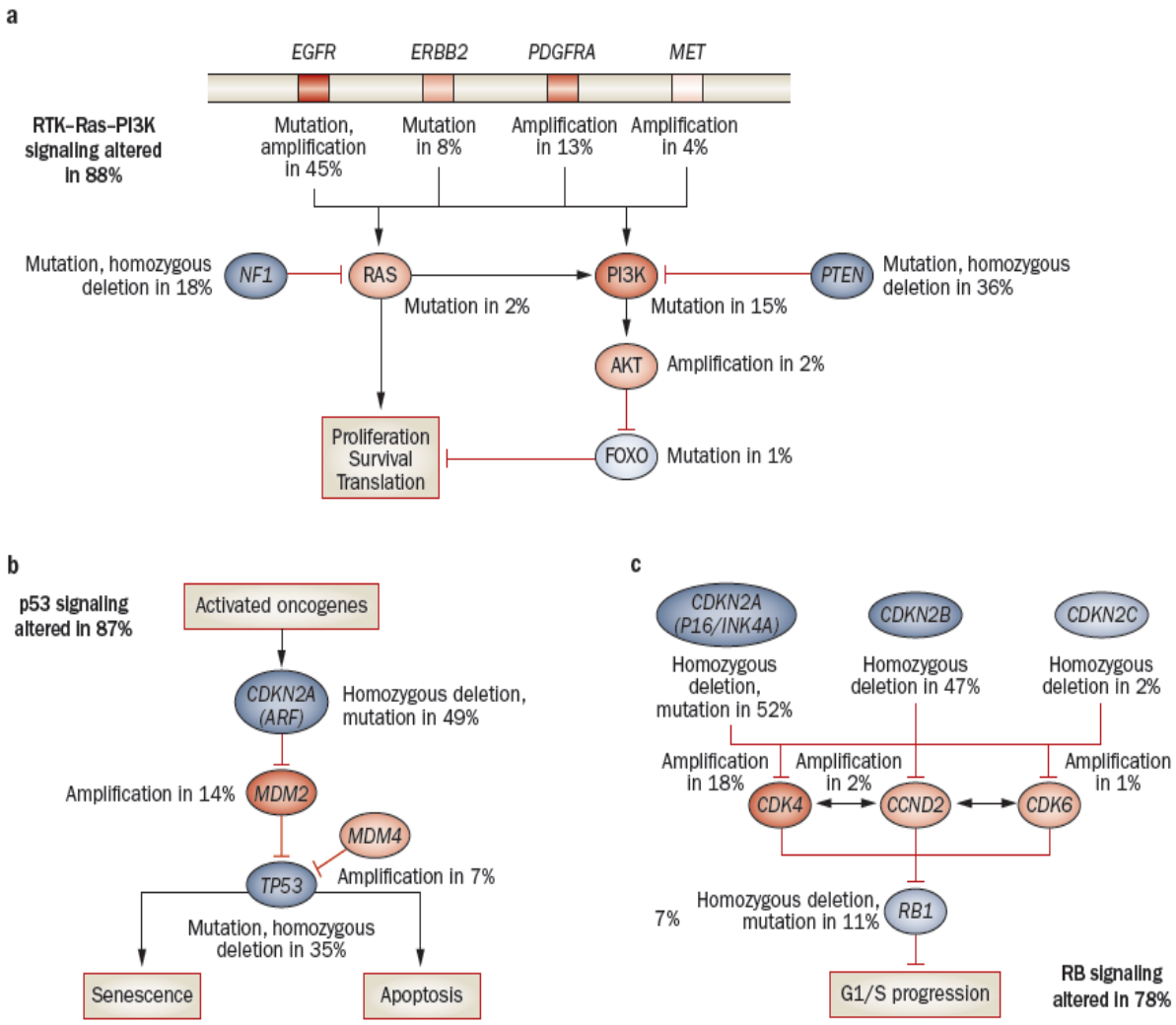


Figure 1. Genomic alterations in glioma involve 3 key signaling pathways. TCGA research network performed large scale genomics analysis of GBM and revealed that alterations occurred in 3 major signaling pathways: in the RTK/Ras/PI3K pathway, in the p53 signaling pathway, and in the RB signaling pathway. This figure was used with permission and originally published by Riddick and Fine (61).

Therapeutics. Malignant gliomas are incurable. Treatment remains palliative, consisting of surgical resection, external-beam radiation, and chemotherapy. Surgery is the front-line defense depending on the tumor location and is an important factor since the amount of tumor resection strongly predicts patient prognosis and survival (62). To ensure that the maximum amount of tumor is removed without affecting critical functions, surgeons utilize cortical and subcortical stimulation during surgery (63). A tumor infiltrating the primary motor cortex or corpus callosum may allow for only partial resection (7). The infiltrative nature of glioma cells makes complete tumor resection virtually impossible, as individual glioma cells pervade surrounding normal tissue leading to tumor recurrence in new anatomical locations.

The standard of care for newly diagnosed GBM includes concurrent radiation and temozolomide treatment with continued temozolomide. This regimen increased median survival from 12.1 months with radiotherapy alone to 14.6 months with radiation plus temozolomide; the 2-year survival rate was increased from 10.4% to 26.5% with temozolomide (57). Temozolomide was the first chemotherapy drug demonstrated to significantly increase GBM patient survival rates (7). Until this report, carmustine was administered adjuvantly following radiation, although large randomized clinical trials demonstrated a significantly increased survival benefit compared to radiation alone. Despite the moderate success of temozolomide, tumors inevitable recur. There is no standard treatment option for recurrent GBM; generally temozolomide is re-administered, and select patients may undergo additional surgery (7).

Low-grade gliomas are treated less aggressively. Surgery is the front-line therapy, similar to GBM. Although the role of chemotherapy in low-grade glioma is not entirely clear,

typically patients are treated with temozolomide (64). Oligodendroglioma and mixed oligoastrocytoma have demonstrated the most favorable response to temozolomide, with 1p19q combined loss dictating the highest response (65).

The explosion of the genomic and high throughput era has provided the opportunity to tailor treatment to individual tumors. Further, targeting specific molecules that play a key role in tumor development and progression should produce an enhanced response versus non-targeted cytotoxic therapies. Molecular targeted therapies should be less toxic to normal cells, as they theoretically target tumor cells alone. Since the majority of GBMs have EGFR alterations, inhibitors against EGFR are an attractive target for therapy; however, inhibition of EGFR alone does not yield promising results. Similar results were observed in a clinical trial evaluating monotherapy of the PDGFR inhibitor, Imatinib (66). Dr. Ron DePinho's group later reported that activation of multiple RTKs was creating signaling redundancy, leading to the ineffectiveness of single RTK inhibition (45). Many clinical trials have assessed effectiveness of targeted agents, primarily against RTKs as well as vascular endothelial growth factor (VEGF), Akt, Src, mTOR, and integrins (67). Most current clinical trials are adding novel agents to the standard of care treatment. Thus far, few have yielded promising results; however, cilengitide (an integrin αv inhibitor) and bevacizumab (VEGF inhibitor) are currently being evaluated in Phase III randomized trials in combination with temozolomide and radiation (7). Select clinical trials are stratifying patients based on *MGMT* promoter methylation to determine its potential prognostic and predictive value (7), given that retrospective studies have reported better response to chemotherapy in *MGMT* promoter methylated cases. Other clinical trials have evaluated tumor cell vaccines, including an EGFRvIII-targeted vaccine (68) and an autologous dendritic cell vaccine (69).

Other trials are assessing oncolytic viruses (70) and herpes simplex virus-thymidine kinase gene therapy (7).

Mouse models of glioma. Mouse models are vital for glioma research. They provide means to evaluate a broad spectrum of hypotheses in a more biologically relevant manner. Cell culture experiments are performed with relative ease and are important and necessary to examine detailed molecular events that are required to form the basis of animal model experiments. However, appropriate mouse models are needed to reflect the heterogeneity and complexity of glioma in a living organism. Highly relevant biological endpoints such as tumor grade and size, vasculature (which plays critical role in tumors), and survival can all be evaluated in mouse models, but not in cell culture.

Xenografts are the most common type of mouse model and are also the most time and cost efficient. Xenograft experiments involve the implantation of cultured human cancer cell lines, either subcutaneously or orthotopically into immunocompromised mice. There are several advantages to this type of model, such as retaining the ability to manipulate the cells prior to implantation. Subcutaneous xenografts offer easy access to the tumor so that tumor growth (or shrinkage) may be measured over time with calipers without the labor-intensive and costly MRI or other imaging modalities. Additionally, drugs can be more easily injected directly into the tumor, precluding the obstacle of drug delivery due to the blood-brain-barrier. Orthotopic xenografts into the brain allow interaction of glioma cells with brain stromal microenvironment and are more likely to reflect a physiologically relevant setting, but the compromised immunity of these mice does not reflect the true microenvironment. Although xenografts are advantageous, there are several disadvantages. In general, they don’t closely recapitulate human glioma due to the use of cell lines that have been

maintained in a very different environment and are likely to have acquired mutations over time and diverged from their original state (71). Further, orthotopic xenografts don't have the distinct diffuse growth patterns that are characteristic of human glioma; xenografts typically produce larger tumors with defined borders. Finally, it is evident that better models are required, since drug development testing has yielded limited predictive values (72).

Genetically engineered mouse models (GEMM) possess much potential for delineating the molecular pathogenesis of glioma development and progression, examining the cell(s) of origin, as well as for preclinical testing of drug candidates. GEMMs enable the study of glioma in a more physiologically relevant environment in immunocompetent mice, with the brain stroma and microenvironment intact. These models are valuable because they allow one to determine the functional roles of key molecules and signaling pathways leading to tumor formation and progression in a living organism. Several studies have reported that the molecular characteristics of tumors derived from glioma GEMMs closely resemble the human counterpart tumors (73-76).

Conventional GEMMs include transgenic and knockout mice. Transgenic mice express transgenes under the control of a specific promoter, which directs expression of the oncogene to a certain cell population or tissue. Tumor suppressor genes may be eliminated via conventional gene knockouts, in which one or both alleles are knocked out in every cell (77). Further genetic manipulation can be achieved by knocking out genes conditionally using Cre/loxP technology, in which loxP recombination sequences are inserted (flanking) on either side of the gene (floxed) (78). Cre-recombinase then mediates recombination and effectively eliminates the gene's function. Floxed mice are crossed with Cre-transgenic mice, which results in recombination. Cell lineage-specific gene knockout may be achieved

by placing *Cre* under promoters such as the glial fibrillary acidic protein (GFAP) promoter, which directs expression of glial precursors and astrocytes. Alternatively, studies have been performed in which Cre was delivered stereotactically into the brain via adenoviral or lentiviral vectors.

Combined transgenic or knockout mice are utilized to study the combinations of important genetic lesions. This approach requires labor-intensive germline mutagenesis and extensive breeding schemes. An alternative method involves viral somatic gene transfer which is generally injected directly into the brain. Initially, murine retrovirus (MoMuLV)-mediated delivery of the PDGFB oncogene was utilized to successfully generate glioma (79). A similar approach involves using the avian retrovirus Replication-Competent Avian leukosis virus long terminal repeat with a Splice acceptor (RCAS), to enable gene delivery (80). Transgenic mice have been engineered to express the RCAS receptor, tv-a, under the control of specific promoters, including GFAP, nestin (directs expression of neural/glial stem cells), or most recently 2′,3′-cyclic nucleotide 3′-phosphodiesterase (CNP) (directs expression of oligodendrocyte progenitor cells) (81). These transgenic mice are referred to as Gtv-a, Ntv-a, or Ctv-a, respectively. RCAS viral particles carrying the transgene are injected into the brains of newborn Gtv-a, Ntv-a, or Ctv-a mice. RCAS infects only cells that express the tv-a receptor, thereby directing transgene expression to a specific cell population (80, 82). RCAS vectors have also been modified to incorporate a Tetracycline (tet) inducible component (83). An RCAS vector containing the reverse tetracycline transcriptional transactivator in addition to an RCAS vector (that places the gene of interest under the control of the tet response element) are injected, enabling timed expression of the gene of interest upon exposure to the tamoxifen analog, doxycycline. The RCAS/tv-a model is advantageous since combinations of oncogenes may be delivered

simultaneously, affording relative ease of examining oncogene cooperation. Further, RCAS infection and subsequent oncogene expression is restricted to a few cells within the injected geographical area, compared to the widespread transgene expression in traditional transgenics. This restricted expression of oncogenes more closely recapitulates the human disease, in which it is generally accepted that tumor formation occurs in a single cell of clonal origin (84).

Most GEMMs have focused on the key signaling pathways that have been reported in human glioma, such as loss or mutation of tumor suppressors *p53*, *PTEN*, *NF1*, and *INK4a/ARF*; and gain of function oncogenes *EGFR*, *PDGF* (*PDGFR*), genes in the PI3K pathway, and the ras gene family, *HRAS* and *KRAS* (84). Typically a single mutation or genetic alteration is insufficient to initiate glioma development, however, there are a few exceptions including *PDGFB*, *HRAS and KRAS*, and inactivation of *RB* (85). As previously mentioned, PDGFB delivered via MoMuLV led to oligodendroglioma (79). Similarly in the RCAS/Ntv-a model, PDGFB consistently yields high-penetrance low-grade glioma resembling oligodendroglioma (86-89). An activating mutation in Ras (V12Ras) induces astrocytoma with histology and grade dependent on gene dosage (74). Finally, inactivation of Rb via truncated SV40T antigen under the GFAP promoter led to 100% tumor penetrance of low-grade astrocytoma by 10-12 months (90).

Combined loss of two or more tumor suppressor genes is sufficient to induce glioma. Loss of both *NF1* and *p53* is an established model of astrocytoma and spans all tumor grades (73, 91). Loss of a tumor suppressor gene combined with expression of an oncogene may also cooperate to induce glioma. While EGFRvIII alone was not able to induce glioma in the RCAS/tv-a system, EGFRvIII combined with loss of *INK4a/ARF*

produced glioma-like features (82). Further, activated Kras with loss of *PTEN* led to glioma (92), as did the loss of *INK4a/ARF* with either v-erbB (activated variant of EGFR) (93) or PDGFB (86, 94). Typically, upon inactivation of an additional tumor suppressor gene, tumor latency is decreased and histological grade is increased (84).

Oncogene cooperation has also been widely studied, particularly in the RCAS-tv-a model, due to the facilitation of injecting various combinations of oncogenes simultaneously. Akt combined with either activated Ras (88, 95) or Raf (96, 97) induced high-grade glioma, including GBM. Additional oncogenic cooperation has been reported with PDGFB and IGFBP2; Kras, IGFBP2, and Akt (88); and Kras, Akt, and c-myc (98).

Investigating the specific glial cell type leading to glioma has recently been investigated (99). Initial reports came from Eric Holland's group where they observed differences in glioma incidence and histology between Gtv-a and Ntv-a mice injected with Kras and Akt. High-grade gliomas developed in Ntv-a mice, however, no tumors developed in either Gtv-a (95) or Ctv-a mice (100), indicating that glial cell subtype plays an important role in the tumorigenic potential of various genetic alterations. Other cell types that have been investigated are neural and glial progenitor cells that inhabit the subventricular zone (SVZ) (101), along with neural stem cells (102, 103). Many studies have reported stem cells as the cell of origin, given the evidence that cells with transgene expression and/or tumor suppressor gene inactivation in and around the SVZ develop glioma, whereas the same genetic mutations in cells in other areas others don't. Lentiviral vectors carrying oncogenic Ras or activated Akt (under the control of *Cre*) induced GBM when injected into the SVZ, but tumors were rarely observed upon injection into areas such as the cortex (104). Further, injection of adeno-Cre into the SVZ of mice with disrupted *NF1*; *p53*; *NF1* and *p53*; *PTEN*

and *p53* resulted in 100% penetrance when injected into SVZ, but not when injected into cortex or striatum (105). Another study found that mutated cells migrated away from the SVZ, suggesting that these stem cells were responsible for glioma development (73). There are other reports, however, that dispute this theory. A recent study examined the inhibitor of DNA-binding (*ID*) genes which maintain self-renewal and multipotency of adult neural stem cells. Cells with low expression of *ID1*, which had high proliferative ability and low self-renewing potential, actually formed tumors with a higher penetrance than cells with high *ID1* expression, which had both high proliferative ability and high self-renewing potential. Disruption of the *ID* genes had minimal effects on animal survival, but knockdown of *Olig2* (committed oligodenrocyte lineage) had a significant survival effect in cells with low *ID1* expression (106). This study indicated that self-renewal potential does not impact tumor growth, demonstrating that glioma initiating cells cannot be identified merely by self-renewal, a hallmark of cancer stem cells.

Mouse models are also of critical importance in preclinical testing, in particular those involving molecular targeted therapies (107). Glioma mouse models have been reported to have a similar disrupted blood-brain-barrier as humans, which is important given the challenge of efficient drug delivery in glioma (108). These models may also be monitored similar to humans. McConville and coworkers (109) reported that gliomas generated from the RCAS/tv-a model could be monitored (and graded) via MRI following temozolomide treatment. Although no animal model will perfectly represent the human counterpart disease, GEMMs can help us to better understand the underlying pathogenesis of glioma and develop logically devised therapeutics.

Insulin-like growth factor binding protein 2

Introduction. Insulin-like growth factor (IGF) binding protein 2 (IGFBP2) is one of six proteins that comprise the IGFBPs, which bind IGFs (IGF-I and IGF-II) with high affinity (110). IGFBP2 is located on chromosome 2 region q33-q34 (111) and is highly expressed during fetal development, expressed at low levels after birth, and highly expressed again throughout puberty (112). IGFBP2 is the second most abundant IGFBP in serum, following IGFBP3 (111), and is the major IGFBP in both cerebral spinal fluid and the brain (113). IGFBP2 expression closely corresponds with IGF-II expression in specific anatomical regions in the brain. Further, IGFBP2 and IGF-II are frequently observed at locations separate from the cells that express these proteins; therefore, it has been suggested that IGFBP2 plays an important role in transporting IGF-II (114). IGFBP2 appears to be involved in various CNS conditions, as there is increased IGFBP2 expression during wound repair, in both activated microglia (indicative of immune response) and astrocytes, in damaged neurons, and in malignancies (115).

Transgenic mice expressing IGFBP2 under control of the robust cytomegalo virus promoter resulted in a postnatal weight reduction, due primarily to affected skeletal composition (116). IGFBP2 mouse knockout studies revealed no overall weight changes, although the liver and spleen weights were increased and decreased, respectively (117). Subsequent studies of *IGFBP2*$^{-/-}$ mice indicated bone abnormalities (118). Other IGFBPs were found to compensate for the loss of IGFBP2 and likely allowed for functional redundancy resulting in fewer developmental defects.

Role in IGF system. The role of IGFBP2 in the IGF system is complex. IGFs are potent mitogens that, upon receptor binding, induce cell proliferation and promote anti-

apoptotic signals. Type I and type II IGF receptors (IGF-IR and IGF-IIR) differ in structure and function (110). IGF-IR is responsible for transducing IGF signaling and forms a tetramer composed of two α and two β subunits. The α subunits are extracellular and bind both IGF-I and IGF-II, thereby initiating IGF-IR kinase activity and subsequent activation of the Ras and PI3K/Akt pathways (119). In contrast, IGF-IIR is a monomer with no tyrosine kinase activity. The extracellular domain binds only IGF-II, but may also bind mannose 6-phosphate proteins and transforming growth factor β. IGF-IIR has been proposed as a possible tumor suppressor gene, since it binds and degrades IGF-II (120). In this manner, IGF-IIR acts as a competing decoy receptor, which reduces IGF-IR activity.

In accordance with the other IGFBPs, IGFBP2 binds both IGFs (although IGF-II with stronger affinity) (121). This interaction regulates the bioavailability of IGFs and may either enhance or inhibit IGF functions. IGFBP2 can perform this role by transporting IGFs to specific cell types or tissues, by stabilizing IGFs thus preventing their degradation, by positioning IGFs in proximity to the receptors via extracellular matrix (ECM) binding, by sequestering IGFs, and finally by transporting IGFs away from their respective receptor (110, 115, 122). IGFBPs are also susceptible to proteolysis, which results in IGF release. Rorive and colleagues (123) demonstrated that the IGFBP2/IGF-II complex could be proteolyzed by matrix metalloproteinase-9 (MMP-9), thereby releasing IGF-II. This was found to contribute to enhanced growth and motility of LN229 astrocytoma cells. Generally, IGFBP2 is thought to have an inhibitory function toward IGFs (122). Overexpression of IGFBP2 has been reported to decrease cell proliferation in human embryonic kidney cells; this effect was abrogated upon addition of exogenous IGF-I (124). In a similar study, the addition of IGFBP2 abolished the IGF-I proliferation effect (125). Further, growth was increased upon knockdown of IGFBP2 in intestinal epithelial cells (126), and the growth

effect in a transgenic mouse line containing high IGF-I levels was reversed upon crossing to IGFBP2 transgenic mice (127).

IGF-independent functions. Beyond the IGF-dependent functions, IGFBP2 also performs multiple independent functions (not involving IGFs). These functions occur via structural domains in the C-terminus, including the heparin binding domain (HBD) and Arginine-Glycine-Aspartic acid (RGD), a known integrin-binding domain (128). Binding of ECM components glycosaminoglycans, heparin, and proteoglaycans have been reported (129-132). A more recent study demonstrated that ECM binding is mediated via the HBD, thereby regulating neuroblastoma growth and invasion (125). Similarly, Kawai *et al*. (128) demonstrated that the HBD was responsible for maintaining bone mass.

The integrin binding function of IGFBP2 is the most established IGF-independent role. Interaction with integrin $\alpha5\beta1$ and $\alpha v\beta3$ are mediated via the RGD domain. Cell surface binding in a Ewing's sarcoma cell line was specific for integrin $\alpha5\beta1$, which decreased cell adhesion and increased cellular migration (133). Our group also observed an RGD-dependent interaction between IGFBP2 and integrin $\alpha5\beta1$ that led to increased glioma cell migration via activation of Rac (134) and JNK (135). In contrast, an interaction between IGFBP2 and integrin $\alpha v\beta3$ in the breast cancer cell line MCF-7 resulted in a negative effect on migration and tumor growth (136). These functional differences may reflect integrin- and cell-specific mechanisms, although this interaction was not dependent on the RGD domain. This may suggest that a specific IGFBP2/integrin interaction via the RGD domain is critical in mediating cell migration.

IGFBP2 in cancer. Elevated IGFBP2 expression has been observed in multiple malignancies, including GBM (137-140), ovarian (141, 142), pancreatic (143), gastric (144), prostate (145), colon (146), breast (147, 148), and various sarcomas (149, 150). IGFBP2 levels increase according to tumor grade in various cancers and serve as an independent prognostic factor (151-153). Importantly, IGFBP2 has been suggested to be a biomarker, as it's been found in cerebrospinal fluid from patients with pediatric medulloblastoma and ependymoma, as well as pediatric acute lymphoblastic leukemia (154, 155). Beyond correlative studies where IGFBP2 has consistently been linked with tumor progression, the most direct evidence that IGFBP2 is a *bona fide* oncogene was reported by our group, where we found that IGFBP2 could directly promote glioma progression in the RCAS/Ntv-a model of PDGFB-induced glioma (88).

Migration and invasion are the most characterized phenotypes associated with IGFBP2. IGFBP2-mediated invasion has been reported in glioma (156), ovarian (157), and human bladder cancer cells, the latter contributing to lymph node metastasis (158). A cDNA microarray analysis revealed up-regulation of a variety of cell adhesion, migration, and invasion genes in primary human GBM cells (159) and in IGFBP2 over-expressing neuroblastoma SK-N-SHEP cells (160). IGFBP2 has been reported to promote invasion in GBM via CD24 (161), and overexpression in SNB19 GBM cells led to upregulation of genes including MMP-2, integrins α5 and α6, fibronectin, and thrombospondin (156). This study demonstrated that MMP-2 is an important mediator of IGFPB2-induced invasion and revealed significant correlation between IGFBP2 and MMP-2 expression (156). Parallel evidence was detected in human GBM tumor cells following bevacizumab treatment. In a subset of GBMs that did not respond positively to bevacizumab, the marked infiltrating cells near regions of angiogenesis were strongly immunoreactive to IGFBP2 and MMP-2 in

immunohistochemstry studies (162). Similarly, in our *INK4a/ARF*$^{-/-}$ PDGFB-induced glioma mouse model, endogenous lGFBP2 was elevated and localized to the tumor invasive front, again providing compelling evidence that IGFBP2 promotes tumor invasion *in vivo* (94). Finally, migration and invasion inhibitory protein (MIIP), a protein involved in inhibiting migration, antagonizes IGFBP2 resulting in abrogation of IGFBP2-mediated glioma cell invasion (163). Interestingly, the chromosomal location of MIIP is often lost in cancers, providing a potential mechanism by which IGFBP2 promotes an oncogenic phenotype.

In accordance with its oncogenic function, IGFBP2 has been linked to important tumor suppressor genes. An inverse relationship between IGFBP2 protein expression and PTEN has been demonstrated (128, 164-167), as well as INK4A and ARF (94). The PTEN inverse relationship was suggested to be functional, as loss of *IGFBP2* abrogated Akt-driven transformation (166). In prostate cancer cells, IGFBP2 induced phosphorylation of PTEN (indicating inactivation), which was dependent on IGFBP2/integrin binding (167). Consistently, Perks *et al*. (165) demonstrated that IGFBP2, when free of IGF-II, can suppress PTEN in an integrin-dependent manner. Our lab also demonstrated that inhibition of IGFBP2 via an antisense approach promoted survival in *INK4A/ARF*-/- mice with PDGFB-initiated glioma, indicating that IGFBP2 was responsible, in part, for mediating effects of *INK4a* and *ARF* loss (94).

Angiogenesis is a crucial aspect of cancer and has been included as a cancer hallmark (168). IGFBP2 has also been linked to angiogenesis, a characteristic feature of GBM, in which increased IGFBP2 led to up-regulation of VEGF and transactivation of its promoter (160). This effect was dependent on IGFBP2 nuclear localization, supporting an IGF- and integrin-independent mechanism. In support of increased VEGF transcriptional

activity, both Wang *et al*. (156) and Azar *et al*. (160) found that IGFBP2 expression led to an increase in hypoxia inducible factor 1 α (HIF1α), a known transcriptional activator of VEGF. Recently, IGFBP2 was found to regulate endothelial cell recruitment via enhancement of IGF-I/IGF-IR binding in a breast cancer metastasis model (169). Therefore, it appears that IGFBP2 promotes angiogenesis through both IGF-dependent and IGF-independent mechanisms.

Cancer stem cells are postulated to be a small population of cells responsible for maintaining the tumor bulk and a major contributing factor in tumor relapse (170). New research has identified IGFBP2 as an important factor in the maintenance of stem cells. The first report demonstrated that IGFBP2 could strongly influence expansion of mouse hematopoietic stem cells (HSC) *ex vivo* (171). Similar results were observed in glioma stem cells (172), epidermal progenitor cells (173) and human cord blood HSCs, in which IGFBP2 could promote *ex vivo* expansion and transplantation into NOD/SCID mice (174). *IGFBP2* mouse knockout studies revealed decreased HSC cycling, survival, and repopulation capabilities. The HSCs in *IGFBP2*-null mice displayed decreased Bcl-2 (indicative of apoptosis) and increased expression of cell cycle inhibitor proteins. Interestingly, these effects were dependent on the C-terminus of IGFBP2, but independent of both the RGD domain and IGF-1R signaling (175). Furthermore, secreted IGFBP2 also has the capability to recruit hematopoietic stem and progenitor cells, potentially contributing to tumor heterogeneity and severity (176).

IGFBP2 is an attractive therapeutic target, as its oncogenic potential is implicated in a diverse array of malignancies. Although no IGFBP2 inhibitors are clinically available, antisense approaches to IGFBP2 inhibition have demonstrated anti-tumor effects, providing

persuasive evidence that IGFBP2 is a valid potential therapeutic target. A second generation IGFBP2 antisense oligonucleotide drug candidate, OGX-225, reduced tumor volume in a breast cancer (MDA-MB-231) mouse xenograft model (147), and an IGFBP2 antisense approach significantly prolonged survival in PDGFB-driven glioma in *INK4A/ARF*-/- mice (94). An immunotherapy approach was recently performed where either IGFBP2 peptide immunization or adoptive transfer of IGFBP2-competent T-cells resulted in inhibition of tumor growth in an MMTVneu transgenic breast cancer mouse model (177). An additional incentive to therapeutically target IGFBP2 is based on mounting evidence that IGFBP2 contributes to chemotherapy resistance. As mentioned previously, IGFBP2 likely contributes to the increased invasiveness of GBM cells following anti-VEGF therapy (bevacizumab) in humans (162). In breast cancer cells, IGFBP2 has been proposed as an antiestrogen-resistant marker (178), given the evidence that IGFBP2 contributes to trastuzumab resistance via ErbB2 stimulation in an SKBR3 breast cancer mouse model (179). Breast cancer cell lines with high IGFBP2 expression, both *in vitro* and *in vivo*, were resistant to paclitaxel-induced suppression of tumor growth or cell viability; in contrast, cells with low IGFBP2 expression or treated with antisense against IGFBP2 were sensitive to paclitaxel (147). The antiestrogen RU 58,688 agent led to increased IGFBP2 expression in a dose-dependent manner in antiestrogen-resistant breast cancer cells (180). Similarly, in prostate cancer, IGFBP2 expression was increased following androgen ablation, leading to enhanced tumor cell growth (181).

Integrins

Introduction. Integrins are a family of heterodimeric proteins consisting of one α and one β subunit that form a non-covalent association. Eighteen α and 8 β subunits individually dimerize to form 24 heterodimeric members (182). Integrins were originally named in light of their ability to link the ECM to the cytoskeleton. Although originally identified as an anchor point for the cell, subsequent research has unveiled a much more complex role of integrins in cell migration, invasion, proliferation, and survival (183). Mouse knockout studies have revealed the essential role of integrins in development and normal adult physiology; including haemotasis, immune function, tissue maintenance, and repair (184, 185). In particular, loss of integrins α5, αv, α4, α9, or integrins β1 and β8 exhibit the most severe phenotype, where integrin α5 null mice result in embryonic lethality at E10-11 and integrin β1 null mutants die soon after implantation (185, 186).

Integrin ligands. Integrin ligands comprise a large variety of proteins, the most predominant being the ECM proteins. These include vitronectin, fibronectin, collagen, laminin, fibrinogen, fibrillin, tenascin, and osteopontin (187). Integrins may also bind receptors on other cells, soluble plasma proteins, cell adhesion molecules, and microorganisms (183). ECM binding plays a critical role in cell migration by providing the traction necessary for cell movement. Adherent cells require anchorage of the cell for cell survival (188, 189); the term “anoikis,” means “homelessness,” and refers to a type of programmed cell death resulting from inadequate cell ECM attachment (188).

Most integrins have the ability to bind multiple ligands with great specificity. The α subunit provides ligand recognition and is therefore responsible for appropriate ligand binding (187). The specificity may be divided into four main subgroups based on structural

interaction: 1) integrins that bind RGD-containing ligands include αV integrins, two β1 integrins, and αIIbβ3. A vast array of proteins contain the RGD domain, such as the major ECM proteins fibronectin, vitronectin, and fibrinogen (187). Not all RGD-containing proteins permit integrin binding, as the surrounding sequences may affect binding by blocking the RGD sequence which must be available for binding (190); 2) LDV-binding integrins include α4β1, α4β7, α9β1, all β2 integrins, and αEβ7. The LDV recognition sequence is structurally similar to the RGD-binding integrins and includes an alternatively spliced portion of fibronectin (185), in addition to intracellular adhesion molecules (ICAMs) and MadCAM adhesion molecules (191); 3) A-domain β1 integrins are laminin and collagen-binding and include α1, α2, α10, or α11 combined with β1. The A-domain is a triple-helical peptide that relies upon a GFOGER motif for ligand recognition (192); 4) non-αA-domain integrins are the major laminin-binding constituents and include α3β1, α6β1, α7β1, and α6β4 (187).

Integrin signaling pathways. Integrins mediate many important cell signaling pathways and have a unique ability to perform bidirectional signaling. Extracellular integrin ligand binding induces a conformational change that transmits information into the cell, known as outside-in signaling. In addition, signals initiated intracellularly induce another integrin conformational change, leading to increased ligand binding affinity. This is known as inside-out signaling.

Integrin ligand binding induces integrin clustering, resulting in recruitment of large complexes of proteins near the membrane (193). These complexes are referred to as focal complexes which connect the ECM to the cytoskeleton. This creates tensional forces resulting in subsequent morphological changes permissible for cell movement (194, 195). Integrins themselves possess no enzymatic activity, and thus rely upon multiple

cytoskeletal proteins, adapter proteins, and protein kinases to transduce signals appropriately. These proteins form a large complex includes at least 156 proteins and is referred to as the “adhesome” (196). Focal adhesion kinase (FAK) and Src were among the first phosphotyrosine proteins identified to play a role in integrin signaling (183). Upon ligand binding, integrin clustering is induced and FAK is recruited to the focal adhesion complex, where it binds paxillin and talin (197, 198). FAK undergoes autophosphorylation, which creates docking sites for SH2 domain-containing proteins, including the enzymes Src-family kinases (SFK), PKC, Jnk, PKA, Ras, Raf, Erk, and PI3K. These signaling pathways result in cell survival, growth, and motility (198). In particular, the GTPase family of proteins (Rho, Rac, and Cdc42) become activated and mediate actin polymerization leading to formation of the focal complexes (lamellipodia and filopodia) required for cell motility (199). Adapter proteins such as talin, vinculin, paxillin, α-actinin, tensin and the IPP complex (integrin-linked kinse-pinch-parvin), also play a major role by creating signaling platforms and organizing signaling networks both spatially and temporally (198).

Integrin signaling is also often coupled with growth factor receptor (GFR) signaling (200). Specifically, EGFR, PDGFR, VEGFR, and Met (hepatocyte growth factor) cooperate with integrin activation to transduce signals within the cell. Growth factors can alter integrin avidity and amplify integrin signaling, while integrin engagement can further activate the aforementioned GFRs (183). In fact, integrin activation is required for ERK signaling (201, 202). Futhermore, evidence suggests that integrins and GFRs can modulate expression of one another (203, 204). This relationship is remarkably important, as both integrins and growth factor signaling are major participants in cancer-related functions.

Integrin activation. Integrins exist in various conformations, which reflect their affinity and activation state. Integrins are typically found on the cell surface in a "resting state," described by a bent conformation with low binding affinity. In contrast, the activated integrin adopts an extended conformation, with high ligand binding affinity (205).

The unique integrin structure governs its function. The overall structure consists of the extracellular portion or "legs", which account for the majority of the protein, a transmembrane domain, and a short cytoplasmic tail. More specifically, the extracellular portion of the α subunit has a β propeller head followed by one thigh and two calf domains. The β subunit consists of a plexin-semaphorin-integrin (PSI) domain, which is a hybrid domain that contains a β-I domain, four epidermal growth factor (EGF) molecules, a β-tail, a transmembrane domain, and a cytoplasmic tail (206). Upon activation, the integrin shifts conformation from a bent state to an intermediate conformation, characterized by transmembrane and leg separation between the α thigh subunits and the β EGF subunits. Finally, complete separation of the leg, transmembrane and cytoplasmic tail domains occurs, resulting in an upright and extended conformation (207). In addition to electron microscopy studies and recent FRET analyses, the availability of conformation-sensitive antibodies has enabled detection of activated integrins and has greatly aided in the current molecular understanding of integrin activation (183).

Integrins monitor the extracellular environment and serve as mechanosensors that become activated via tensional forces and mechanical stress (193). Regardless of whether signaling is initiated outside or inside the cell, the β cytoplasmic tail is crucial in transducing integrin signaling. Key intracellular proteins bind this region, and include talin, kindlins, and filamin (184, 208-210). Among these, talin is well characterized and is sufficient to activate

β1 and β3 integrins (211, 212). Talin exists in both an inactive (folded) and active (extended) form. Its structure includes a smaller head and a helical rod (213). The head contains a FERM (band four-point one, ezrin, radixin and moesin) domain that is required to bind the cytoplasmic tail of β1 (214). Talin may be activated via a guanine nucleotide exchange factor (GEF)/GTP-protein cascade, which is modulated through G-protein coupled receptor (GPCR)-mediated increase in Ca^{2+} and diacyl glycerol (DAG). This pathway involves RAP1 and RIAM, which binds talin and results in a conformational change (206). Talin is then recruited to the plasma membrane where the third FERM subdomain (F3) directly binds the integrin β1 cytoplasmic tail (215). This association is thought to destabilize integrin transmembrane and cytoplasmic interactions, leading to an extended conformational shift (215). Talin may also be activated via cleavage by calpain, which exposes the F3 subdomain and enables integrin binding (216). Further, phosphatidylinositol (4,5)-biosphosphate (PIP2) levels help mediate an association with the integrin tail (213).

Integrin specificity and activation may be controlled through various mechanisms, including the regulation of the local amount of talin via PIP2, Rap1, and calpain (206). Further, differential β integrin affinities for talin (217) and the phosphorylation state of β integrin (particularly β3 integrin) contribute to the degree of integrin activation (206). Integrin recycling and trafficking is an additional component that regulates integrin availability and activation (218). Integrins continually undergo endo/exocytic trafficking, where they are endocytosed and taken back to the membrane or degraded via a lysosomal component (219). Integrin trafficking is important for key integrin functions and cooperates with the actin cytoskeleton in the formation of new adhesions and lamellipodia (220). Various routes and mechanisms are involved in integrin internalization that are either clathrin-dependent or

-independent (221). The Rab proteins play a dominant role in the endosomal compartment, in addition to ARF6. It has been recently reported that active and inactive β1 integrins are recycled via distinct pathways and kinetics (222). Although the net endocytic rates among active β1 integrin are higher, inactive β1 integrins are returned to the plasma membrane more quickly. The steady-state condition indicates that inactive β1 integrins are more often present at the cell membrane, whereas active β1 integrins are mostly in the intracellular compartment. The authors suggested this likely indicates that inactive integrins are targeted to areas in which they would be available for activation, such as lamellipodial protrusions (222).

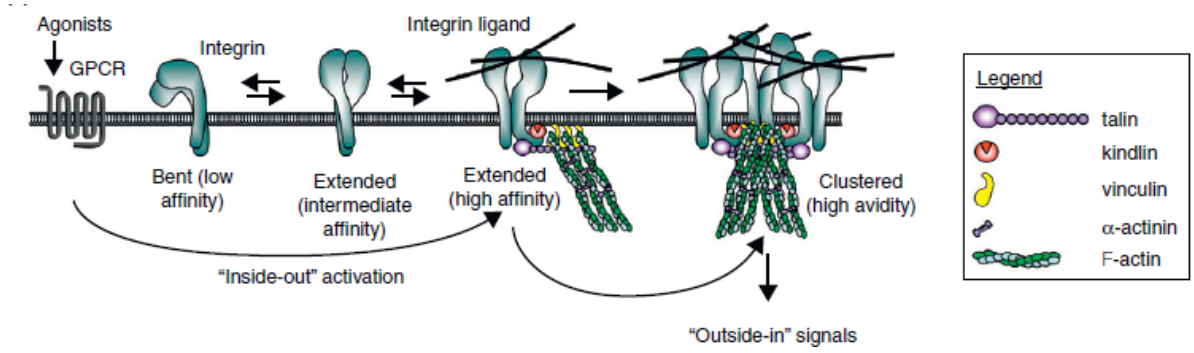


Figure 2. Schematic of integrin activation. Integrins shift from a bent conformation to an extended conformation upon either an inside-out activation or by an outside-in mechanism that involves extracellular ligand binding. Originally published by Margadant *et al*. (221). Published with permission.

Integrins in cancer. Given the essential role of integrins in normal processes such as cell migration, invasion, proliferation, and survival, it is not surprising that these functions are exploited in the development and progression of various malignancies. Integrins are commonly overexpressed in cancer. Specifically, integrins αvβ3, α5β1, and αvβ6 are overexpressed and correlated with decreased survival in a variety of tumors; αvβ5, α6β4, and α4β1 also are associated with decreased survival, although other integrins also play important roles in cancer (223).

Breast cancer mouse models have made important contributions to the integrin field. Conditional integrin β1 knockout in the MMTV-driven polyoma virus middle T antigen (PyVmT) mouse revealed that β1 integrins were required for tumor initiation. Tumors with disrupted integrin β1 exhibited decreased proliferation and altered activation and subcellular localization of FAK (224). Loss of FAK in the PyVmT mouse reduced proliferation of transformed cells and prevented malignancy and metastasis (225), indicating that FAK plays a crucial role in integrin β1 breast tumor progression. In a mutant K-ras (G12D) mouse model of non-small cell lung cancer (NSCLC), loss of integrin α1 attenuated tumor incidence and size and promoted longer survival compared to K-ras-derived tumors with wild-type integrin α1 (226). These findings suggest a pivotal relationship between integrin α1β1 in NSCLCs with mutant K-ras. Receptor transactivation and crosstalk of RTKs and integrins contribute to tumor initiation and progression. Integrins β1 and β4 cooperate with ErbB2 in the MMTV-driven PyVmT model of breast cancer; disruption of either β integrin led to a marked decrease in metastases, delayed tumor initiation, and down-regulated integrins α6 and β5, in comparison to ErbB2-driven tumors with wild-type integrin (227, 228). Although many integrins contribute to tumorigenesis and progression, some may serve as

negative regulators. Loss of integrin $\alpha 2\beta 1$ in primary epithelial tumor cells led to increased cell migration, intravasation, and anchorage-independent growth. Moreoever, metastasis was promoted in PyVmT mice with integrin $\alpha 2\beta 1$ and activated ErbB2 (229), while $\alpha v\beta 8$ integrin has been reported to suppress angiogenesis in a mouse model of glioma (230).

Altered expression of signaling adapters and ECM components play a major role in tumorigenesis. Glioma cells aberrantly express tenascin, vitronectin, hyaluronic acid, and osteopontin (231). Many integrin adaptors, such as p130CAS, NEDD9, CRK, p140CAP, integrin-linked kinase (ILK), PINCH1/2, and Parvin-β are also overexpressed in various tumors, and mouse models of breast cancer have revealed their important contribution to integrin signaling (198). MMTV-driven *Bcar1* (p130CAS) induces mammary gland hyperplasia during pregnancy, and decreases tumor latency when combined with ErbB2 (232). Knockout of *Nedd9* in MMTV-PyVmT mice delayed tumor onset and reduced tumor size (233), while expression of CRK under MMTV control led to a low incidence of mammary epithelial tumors (234).

Therapeutic inhibition. Integrin inhibitors have been pursued in preclinical models and clinical trials. Preclinical studies found that integrin inhibitors targeted both tumor and tumor-associated cells, such as the vasculature endothelium (223). Several clinical trials are ongoing, including function-blocking antibodies against integrin $\alpha v\beta 3$ (etaracizumab) (235), αv integrin-specific monoclonal antibody (CNTO 95) (236), anti-integrin $\alpha 5\beta 1$ function-blocking antibody (237), and a small peptide antagonist against integrin $\alpha 5\beta 1$ (ATN-161) (238). The most successful integrin antagonist has been cilengitide, a cyclic RGD peptide that targets integrins $\alpha v\beta 3$ and $\alpha v\beta 5$ (239). Preclinical studies in an orthotopic model of glioblastoma demonstrated its effectiveness in inhibiting tumor growth and

angiogenesis (240, 241). Subsequent phase I trials showed that it was well tolerated with few toxicities (242, 243). Separate phase II trials were performed in recurrent GBM or newly diagnosed GBM. Recurrent GBM demonstrated anti-tumor effects (244), whereas the newly diagnosed GBM reported a 69% 6-month progression-free survival (PFS), which rose to 91% in patients with *MGMT* promoter methylation (245). Currently, the first phase III trial in assessing an integrin antagonist is ongoing. This trial will evaluate survival among patients with promoter-methylated *MGMT* who are administered cilengitide in combination with temozolomide and radiotherapy (223).

Given the accessibility and tumor-specific overexpression of integrins, they are currently being utilized as a target for drug delivery and tumor imaging in preclinical mouse models. Examples include MRI-imaging nanoparticles that target specific integrins (246) and RGD radio-labeled peptides (247-249). Integrin $\alpha v\beta 3$ has been imaged in human tumors via scintigraphic imaging using a radiolabeled integrin-targeted peptide (250, 251). Radiolabeled peptide (18F-galacto-RGD) with positron emission tomography (PET) has also been utilized, which enabled quantitative assessment of integrin $\alpha v\beta 3$ (252).

Integrin-linked kinase

Inroduction. ILK was first discovered as an integrin-interacting protein via a yeast-two hybrid screen for proteins that interacted with the cytoplasmic tail of integrin β1 (253). Subsequent work has extended this interaction to β3 integrins (254). As part of the integrin complex, ILK is essential in transducing a variety of signaling pathways that are important in both normal physiology and in cancer processes. Under normal conditions ILK is involved in cell adhesion, tissue homeostasis, and of particular importance in performing critical cardiac functions. Phenotypically, ILK knockout mice are embryonically lethal (E5.5-6.5) (255) and display a very similar phenotype as integrin β1 knockouts (186). Conditional loss of ILK in specific tissues has revealed various developmental defects, including dwarfism and chondrodysplasia upon ILK deletion in chondrocytes (256, 257); dilated cardiomyopathy upon ILK loss in cardiomyoctes (258); and impaired vascular development and subsequent embryonic lethality upon loss in endothelial cells (259).

The basic structure of ILK consists of an N-terminal domain containing four ankyrin (ANK) repeats, a central plekstrin homology (PH) domain, and a C-terminal kinase domain (260). These domains mediate protein interactions in which ILK serves as both an important scaffolding protein and as a kinase, thereby activating key cell survival and motility pathways. ILK plays a critical role in focal adhesion complexes and is the central protein in a tripartite complex. These proteins include PINCH, which binds the ANK domain, and parvin which binds the kinase domain (261). This complex is commonly referred to as ILK-PINCH-parvin (IPP). Multiple connections to the cytoskeleton are facilitated by α and β parvins, which can interact with actin directly; by paxillin, which binds ILK and facilitates an actin connection through vinculin and actopaxin binding; and via Mig binding, which

mediates an actin linkage through migfilin and filamin (260). Through these interactions, ILK links integrins to cytoskeletal components and thus regulates cell spreading and motility. ILK also couples integrin and GFR signaling through the tyrosine kinase receptor adapter protein, Nck (binds ILK indirectly via paxillin). A complex involving ILK, paxillin, and Nck is recruited to EGFR and PDGFR upon receptor stimulation (262). In this manner, ILK can further transduce GFR signaling as well as mediate cell motility.

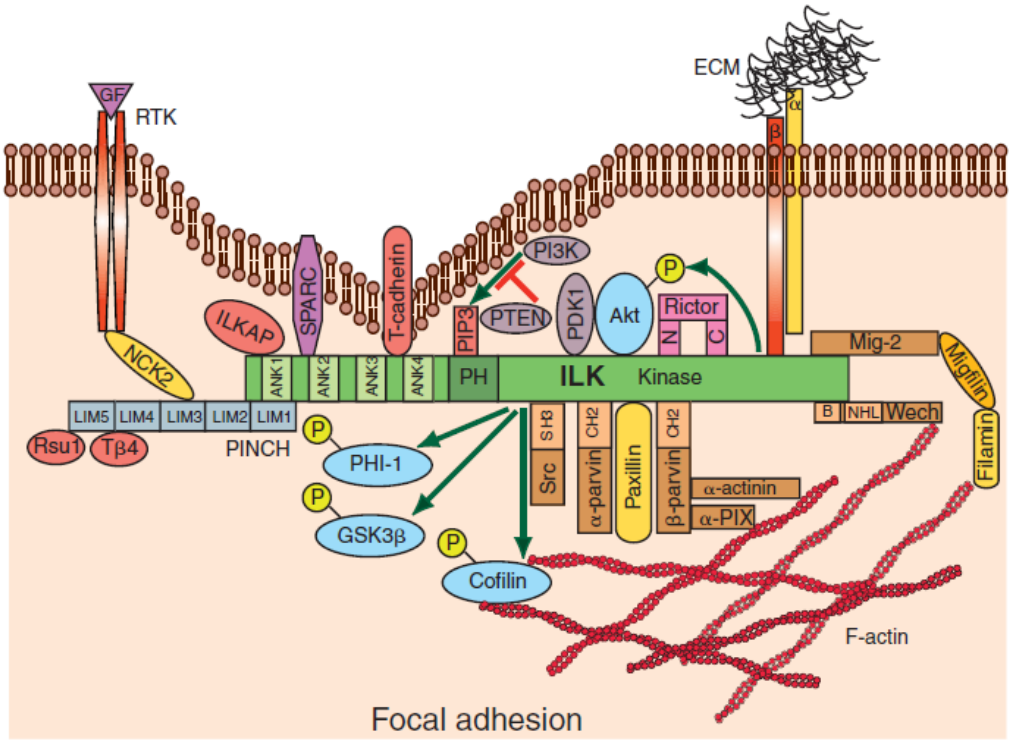


Figure 3. ILK is a critical signaling molecule. ILK is both a kinase and an adaptor protein that couples integrin and growth factor signaling. Originally published by McDonald *et al*. (260). Used with permission.

Kinase or pseudokinase? ILK serves both as an adapter protein and as a serine/threonine kinase. ILK activity is stimulated by integrins, growth factors, and chemokines. *In vitro* assays have identified at least 12 ILK substrate proteins, including integrins β1 (253) and β3 (254) and among the best-documented include phosphorylation of GSK-3β at Ser9 and Akt at Ser473. Phosphorylation is mediated by PIP3 binding to the PH domain (263) and substrate binding to the kinase domain; disruption of either PIP3 binding (R211A mutation) or Akt binding (S343A mutation) prevents full Akt activation and renders ILK "kinase-dead" (264). Akt requires phosphorylation at both Thr308 and Ser473 for full activation and thereby regulates multiple cancer-promoting functions such as cell proliferation, survival, and angiogenesis (265). ILK phosphorylation of Akt (S473) has been reported to require an interaction with Rictor (266). GSK-3β phosphorylation results in its inhibition and subsequent stabilization of β-catenin, which translocates to the nucleus in conjunction with TCF/Lef to induce transcription of cell proliferation genes such as *cyclin D1* and *Myc* (261). Additionally, GSK-3β regulates *cyclin D1* through cAMP-responsive element-binding (CREB) (267). ILK kinase activity is antagonized by PTEN and ILK associated protein (ILKAP) (265). PTEN blocks PI3K activity, and thus ILK, by reverting PIP3 to PIP2. In support of ILK's PI3K-dependency, PTEN loss leads to constitutive ILK activity in cancer cells (268, 269). ILKAP binds ILK via the N-terminal ANK domain and reduces ILK kinase activity and GSK-3β phosphorylation, but does not antagonize Akt phosphorylation (270, 271), providing an additional level of specificity and regulation. Although multiple investigations have demonstrated ILK kinase activity, much debate has been generated regarding the kinase capabilities of ILK. ILK contains significant homologies to Ser/Thr protein kinases; however, critics cite the observation that ILK lacks classic conserved sequences of typical kinases within the catalytic loop and the DXG motif

(272, 273). Further, the catalytic domain sequence diverges across species, suggesting a non-essential kinase function (261). Advocates argue that several proteins, including Mik1, Vps15p, CASK and haspin, are atypical kinases that were once labeled as pseudokinases, but now have been confirmed as catalytically active (274). An additional argument against the kinase function was that the ILK enzyme kinetics were unknown; however, a recent study reported that the enzyme kinetics were similar to that of classical kinases (275). There is ample evidence already cited of ILK kinase activity; however, efforts to elucidate the ILK kinase activity have been confounded since various mutations which affect kinase activity also perturb important binding partners, making it impossible to determine whether decreased substrate phosphorylation was due to the disrupted complex, or in reality due to loss of kinase activity of ILK (261). For example, Ser343 in the activation loop has been proposed to be autophosphorylated (253). A S343D mutation which mimics phosphorylation resulted in constitutive ILK activation, whereas a S343A mutation which is unable to be phosphorylated, suppressed ILK signaling (264). In contrast, murine developmental studies did not corroborate these findings. The S343A putative kinase inactivating mutation exhibited no development defects, suggesting that the ILK kinase function is dispensable in embryonic development (276). In *ILK* null macrophages, Akt Ser473 phosphorylation was attenuated (277); however, this same attenuation was not observed in fibroblasts or chondrocytes (255-257), indicating there may be cell-type specificities involved.

Hannigan *et al*. (278) suggested that in general, some functions require kinase activity and some are kinase-independent, serving as a scaffolding or adapter protein. A K220M-mutatation was investigated and found to have a disrupted interaction with α-parvin; therefore, the authors concluded that the K220 site was the essential α-parvin binding site, as opposed to a true catalytically active site (279). However, it has been reported that ILK

kinase activity was attenuated upon α-parvin binding (275). Hannigan's group hypothesized that the attenuated kinase activity likely works to keep ILK in an inactive conformation, and that α-parvin binding may be dependent upon ILK kinase activity (278). In support of the importance of this site, mice harboring a K220M mutation died shortly after birth and almost all animals had impaired renal development and function (274). These data suggest that the PI3K-dependency of ILK could be context-specific, as it is not always observed. As mentioned previously, there are indications that ILK kinase activity is more important in cancer-related processes and cell signaling, whereas in embryogenesis and development, the kinase function plays less of a role (278).

ILK in cancer. The role of ILK in cancer is increasingly recognized. ILK is overexpressed in a variety of malignancies and correlates with tumor progression in bladder cancer (280), non-small cell lung cancer (281), ovarian (282), colon adenocarcinoma (283, 284), melanoma (285), prostate (286), and gastric cancers (287). Strikingly, increased ILK expression was reported in 100% of Ewing's sarcoma (288). Overexpression of ILK was initially reported to inhibit adhesion, promote anchorage-independent growth (253), and cell cycle progression via increased cyclin D in rat epithelial cells (289). Further, ILK expression in epithelial cells was demonstrated to induce tumors in nude mice, highlighting its potential as a potent oncogene (290).

Transgenic mice expressing ILK from the MMTV promoter in mice have revealed a direct involvement of ILK in breast cancer development and progression. Overexpression of ILK alone led to a 34% incidence of mammary tumors by an average 18 months (291), and ILK cooperated with Wnt1 to significantly accelerate tumor development (292). In an ErbB2-induced mouse model of breast cancer, loss of ILK dramatically delayed tumor onset, and

pre-malignant lesions exhibited strong growth defects, indicating its critical role in cooperating with GFR in tumorigenesis (293). Mouse models also support an ILK function in colon cancer, as loss of ILK resulted in fewer tumors in a colitis-associated model of colon cancer (294).

Similar to IGFBP2, ILK is involved in angiogenesis. ILK was shown to induce VEGF expression via HIF-1α in epithelial and prostate cancer cells; inhibition of ILK reversed this induction and further, led to inhibition of prostate tumor angiogenesis and tumor growth (295). Similar results were obtained in GBM xenografts, where small molecule ILK inhibition decreased tumor HIF-1α and VEGF secretion, along with blood vessel mass and tumor growth (296). A more recent study found that ILK promoted angiogenesis in melanoma xenografts via induction of IL-6 and nuclear factor-kappa B (NF-κB), likely through Stat3-mediated *VEGF* transcription (297).

The epithelial to mesenchymal transition (EMT) has gained significant attention in cancer research and describes a phenomenon whereby epithelial cells alter gene expression programs toward a mesenchymal phenotype, thus acquiring increased motility and enablement of metastasis (298). The morphology of epithelial cells is not amenable to motility, with tight cell-cell junctions mediated by E-cadherin; mesenchymal cells do not have close linkage to other cells and have a shape amenable to motility (299). EMT gene expression programs are typified by various transcriptional factors, including Wnt, β-catenin, tumor growth factor beta (TGFβ), among others, resulting in decrease of epithelial makers such as E-cadherin, and an increase of mesenchymal markers such as vimentin (298).

ILK overexpression in epithelial cells consistently results in EMT, with decreased E-cadherin levels and cellular morphological changes resembling fibroblasts (290, 300, 301). Importantly, ILK has been shown to induce a full EMT by downregulating epithelial genes, including E-cadherin, cytokeratin 18, and MUC1; while upregulating the mesenchymal genes LEF1 and vimentin (299). The ILK-mediated EMT phenotype has also been observed in the MMTV-ILK mouse model (291). Multiple mechanisms have been reported in which ILK induces EMT. First, GSK-3β activity (degradation of β-catenin) is inhibited via ILK phosphorylation (302), resulting in nuclear translocation of β-catenin/Tcf, a known mediator of EMT via upregulation of mesenchymal genes (303). The Wnt pathway is frequently increased in cancer, and also plays a role in EMT by inhibition of GSK-3β and increased β-catenin levels (304). This situation results in an excess of β-catenin in the free cytoplasmic pool, leading to nucleus translocation with TCF, where it activates transcription of mesenchymal genes such as fibronectin (299). Second, ILK modulates expression of an E-cadherin transcriptional repressor, Snail, which contains a Snail ILK responsive element (SIRE) (305). ILK has been reported to mediate PARP-binding to the SIRE, resulting in increased Snail transcription and subsequent decreased E-cadherin (306). ILK overexpression also led to an increase of Zeb-1, an additional E-cadherin transcriptional repressor (307). Third, interaction of ILK with rictor mediates TGFβ-induced EMT through Snail and Slug (308). Finally, overexpression of ILK in breast cancer cell lines resulted in significant upregulation of Twist, a known repressor of E-cadherin (309), indicating an additional mechanism by which ILK may induce EMT.

ILK is well documented to promote cell survival and growth; however, researchers have recently identified novel mechanisms in these processes. It was first reported that ILK is transported to the nucleus via phosphorylation by PAK1, and functions to maintain

nuclear integrity (310). Surprising ILK functions were uncovered in the nucleus, where ILK associates with tubulin and tubulin-interacting proteins, which assemble mitotic spindles during mitosis (311). This function was further developed when Fielding *et al*. (312) found a critical function of centrosome clustering in cancer cells, thereby protecting cells from cell death due to supernumerary centrosomes. ILK performed this function with ch-TOG and TACC3; inhibiting ILK led to mitotic arrest and cell death in mitosis. This binding function could have potential implications in cell division errors and genomic instability.

Therapeutic targeting. Cancer cells appear to be more dependent on the kinase activity of ILK than their normal counterparts. Pharmalogical ILK inhibition resulted in attenuation of Akt phosphorylation in breast cancer cells, whereas this same effect was not observed in normal cells, including human breast epithelial cells, mouse fibroblasts, or vascular smooth muscle cells. Inhibition of Akt S473 phosphorylation in breast cancer cells was accompanied by induction of apoptosis and decreased expression of mammalian target of rapamycin (mTOR) (313). This makes ILK an even more attractive therapeutic target, as it would potentially be less toxic to normal cells. Indeed, small molecule ILK inhibition in a mouse model of renal fibrosis affected the molecular events leading to excess collagen production, but did not affect kidney structure or function in healthy mice (314). An additional report of a cancer-specific ILK inhibition was found in acute myeloid leukemia (AML) initiating cells, where inhibition decreased survival of AML cells while causing minimal toxicity in normal bone marrow progenitor cells (315). Inhibition of ILK either by RNA interference approaches or by small molecule compounds has been effective in eliciting anti-tumor responses. Stable knockdown of ILK in a melanoma xenograft model significantly reduced tumor growth, indicating the role of ILK in growth mechanisms (316). Another study utilized an antisense therapeutic approach. *In vitro* transfection of ILK

antisense into PTEN-mutant GBM cells induced apoptosis, and daily treatment of ILK antisense molecules led to stable disease in established xenografts in Rag-2M mice, as opposed to untreated mice which experienced more than 100% tumor volume increase (317). Small molecule inhibitors have been developed which compete for ATP binding in the active site and have demonstrated ~100-fold selectivity over related kinases (318, 319). The ILK inhibitor QLT0267 inhibited growth and reduced invasion and VEGF levels in GBM cells (320). Similarly, human squamous cell carcinoma of the head and neck cell lines exhibited decreased cell growth and induction of apoptosis upon QLT0267 treatment (321). This inhibitor has also provided encouraging outcomes in animal models. ILK inhibition resulted in decreased tumor volume, apoptosis in both tumor cells and tumor-associated endothelial cells, and reduced vascular density in thyroid cancer xenografts (322). Importantly, ILK inhibitors have been administered in conjunction with standard chemotherapies and demonstrated synergistic effects. Combined QLT2054 and gemcitabine treatment led to a 5.4 fold increase over single agent therapy in induction of apoptosis in orthotopic primary pancreatic cancer xenografts (323). Further, ILK inhibition with either docetaxel or cisplatin led to increased survival (324) and tumor volume (325) in orthotopic models of breast cancer and NSCLC, respectively. Therefore, there is ample evidence for the multiple oncogenic functions of ILK and for the promise of its therapeutic intervention.

Nuclear factor-kappa B

Introduction. NF-κB, or Rel, comprises a family of transcriptional factors involved in many biological processes, including inflammation, immunity, and cell survival. Its name is derived from initial experiments in which NF-κB bound the enhancer of the immunoglobulin κ light-chain gene in activated B cells (326). Subsequent studies have revealed that the naming scheme is incorrect, as NF-κB is a pleiotropic transcriptional factor present in every cell and is located in both the cytoplasm and the nucleus (327).

There are two classes of NF-κB family members and each contain an N-terminal Rel homology domain (RHD) that mediates DNA binding or dimerization. One class includes RelA (p65), RelB, and c-Rel, which are synthesized in their complete forms and contain a C-terminal transactivation domain. The other class includes NF-κB1 (or p105) and NF-κB2 (or p100), which are synthesized as longer proteins that are subject to ubiquitin-dependent proteolytic cleavage. These family members lack transactivation domains, but contain multiple inhibitory C-terminal ankyrin repeats. Upon proper activation, the proteins are cleaved into their shorter, active forms (p105 to p50; p100 to p52) and form either hetero- or homodimers (328). The 5 NF-κB proteins can form 15 unique transcription factors; 7 of these contain a transcriptional activation domain and thus are transcriptional activators, 3 bind DNA but are not transcriptional activators, and 3 do not bind DNA (329). The prototypical heterodimer is p65/p50 and consequently, the generic term NF-κB generally refers to p65/p50. The multiple heterodimers that form lends itself to the regulation of the diverse biological processes controlled by NF-κB. Although NF-κB dimers bind to 8-10 base pair regions within κB enhancer elements, dimers differ in their affinity for specific DNA sequences (330). Additionally, specificity may be achieved by the cooperation

with additional co-activators for induction of full transcription (331), such as the transcription factors STAT3, HIF-1α, and AP1 (332).

NF-κB regulation and activation. The activation of NF-κB is tightly controlled and includes feedback mechanisms to prevent constitutive activation (333). Three specific inhibitory proteins, inhibitor of κ B (IκB) α, IκBβ, and IκBε, in addition to the C-terminal domains of p105 and p100, closely regulate NF-κB activity via two main mechanisms. These mechanisms are known as the canonical (classical) or the non-canonical (alternative) pathway (329). In the canonical pathway, the IκB proteins bind NF-κB through the RHD, thereby masking the DNA binding sequence and retaining NF-κB in the cytoplasm (327). The non-canonical pathway involves the precursor proteins p105 and p100, in which the C-terminal portion folds, masking the RHD with the inhibitory ankyrin domains (327). A dominant mechanism in termination of NF-κB activity is the immediate NF-κB-mediated transcription of its inhibitor, IκBα, which can dislodge NF-κB DNA binding (334, 335) and shuttle activated NF-κB out of the nucleus (336, 337). NF-κB activity is also modified via extensive posttranslational modification, including ubiquitination, which serves as an additional mechanism to terminate NF-κB activity (338).

NF-κB may be activated by a diverse set of stimuli, including pathogens such as bacterial-derived lipopolysaccharide (LPS), proinflammatory cytokines such as tumor necrosis factor (TNF) and specific interleukins, growth factors including EGF, various environmental hazards (e.g., cigarette smoke) and certain forms of stress, including physical, chemical, and cellular stressors (331). The canonical pathway responds to pathogens and proinflammatory cytokines, and produces a quick and reversible response, independent of protein synthesis. Stimuli bind to appropriate receptors, including toll-like

receptors (TLRs), TNF receptors (TNFR), B-cell receptors (BCRs), and T-cell receptors (TCRs) (339). Upstream signaling events converge upon the IκB kinase (IKK) complex, which includes two catalytic subunits, IKKα and IKKβ, and one regulatory scaffolding subunit, IKKγ. The IKKβ subunit directly phosphorylates IκBα at Ser32 and Ser36, leading to disruption of NF-κB binding and immediate ubiquitination (340). Ubiquitination usually occurs via a β-TrCP complex, which marks IκBα for degradation by the 26S proteasome (328). This process then releases NF-κB where it translocates into the nucleus and binds the κB enhancer of target genes. In contrast to the canonical pathway, the non-canonical pathway produces a slow and chronic NF-κB activation that is dependent upon protein synthesis. Additionally, this pathway produces more specific functions, including lymphoid development, survival and maturation of B-cells, dendritic cell activation, and bone metabolism (339). The non-canonical pathway has some overlapping, but also distinct molecules compared to the canonical pathway. Activation is mediated by TNF family members and induces NF-κB inducing kinase (NIK), which in turn phosphorylates an IKKα homodimer (327). This series of events enables phosphorylation and proteolytic processing of p100, thereby removing the inhibitory ankyrin domains and producing the final product p52, which binds RelA, RelB, or c-Rel to activate transcription (327).

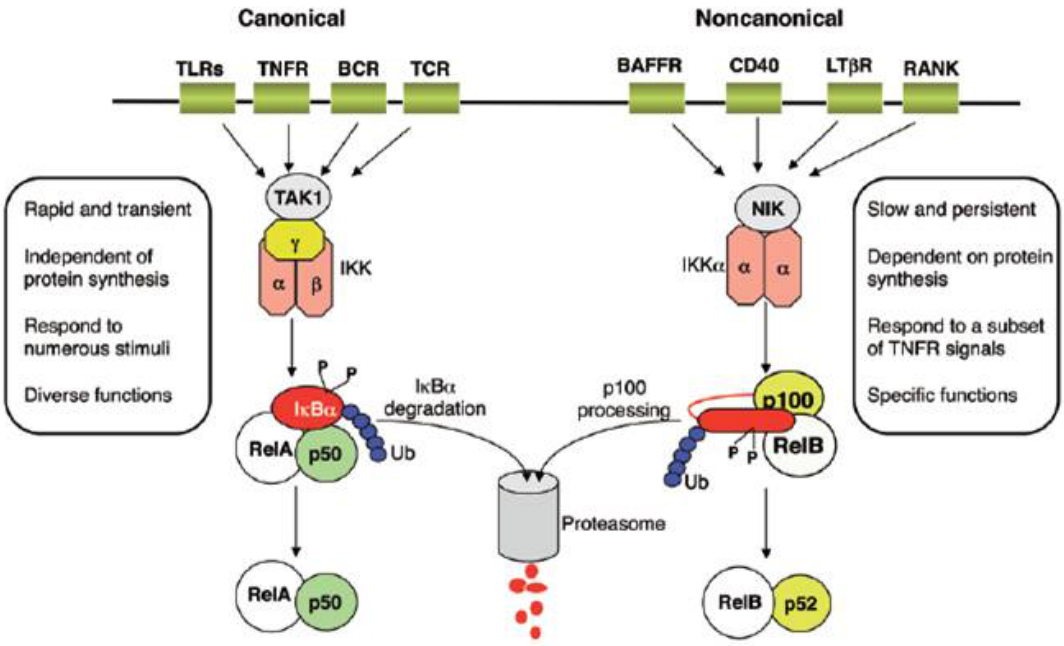


Figure 4. Canonical and non-canonical NF-κB signaling pathways. The canonical and non-canonical pathways involve overlapping, but distinct molecules, and contain distinct differences. Originally published by Sun (339). Used with permission.

Additional mechanisms also exist that induce NF-κB activity independent of IKK or IκBα phosphorylation, some of which include direct phosphorylation of p65 by protein kinase A (PKA) (341) and GSK-3β (342); and tyrosine phosphorylation of IκBα induced by hypoxia and oxidative stress (343) or EGFR activation (344). Further control is governed by posttranslational modifications that modulate DNA binding and interaction with co-activators or co-repressors. Modifications of p65 are extensive and include phosphorylation, acetylation, methylation, and ubiquitination. Specifically, phosphorylation at S276 and S311 increase DNA binding, and phosphorylation at S276, S468, S529, and S536 induce transcriptional activation (332). S536 appears to play a major role, since its phosphorylation

is required for p300 interaction and subsequent transactivation; conversely, S536 is subject to negative regulation via WIP1 phosphatase activity (345). Acetylation at various sites may be either stimulatory or inhibitory, while methylation and ubiquitination are inhibitory (332).

NF-κB and cancer. The first indication of an NF-κB role in tumor promotion stemmed from the report that a viral form of NF-κB (v-Rel) induced avian reticuloendothelial lymphomatosis (346). Given that NF-κB regulates genes involved in cell proliferation, survival, angiogenesis, invasion, and metastasis, it is not surprising that multiple lines of evidence now demonstrate constitutively active NF-κB in most cancer types (332, 347-349). A variety of mechanisms may lead to this aberrant signaling; however, in most solid cancers, genetic alterations or altered expression of NF-κB family members are rare (350). Some have suggested that the autocrine secretion of tumor-promoting inflammatory cytokines is the major contributor of constitutive activation (351). Additionally, loss of TSGs and aberrant expression of GFRs and kinases are mechanisms of inducing NF-κB constitutive activation. For example, the PI3K/Akt pathway is particularly important in activating NF-κB, and is itself activated through many mechanisms, including GFR amplification/overexpression and loss of *PTEN* (332). Finally, pathogen-mediated activation of NF-κB is involved in carcinogenesis, including human papilloma virus (HPV) and *Helicobacter pylori*, which are oncogenic pathogens found to constitutively activate NF-κB (350).

Mouse models have provided valuable insights into the functional complexity of NF-κB in cancer development. TNFα, a potent NF-κB inducer, has been reported as a tumor promoter, most likely via NF-κB (352). Genetic ablation of p65, IKKβ, or IKKγ in mouse models are embryonically lethal and display degenerative liver phenotype due to apoptosis

(353). However, mice lacking other family members display a less severe phenotype, but often have an impaired immune response (327). Conditional ablation of NF-κB family members has allowed investigations of direct roles in cancer development and progression. These studies have revealed that context and cell-type specificities are major determinants of the tumorigenic fate of the NF-κB family members (354). Typically, conditional deletion of IKKβ has reduced tumor incidence and development. This effect has been reported in intestinal epithelial cells, which decreased tumor incidence (355); in myeloid cells, which decreased tumor size (355); and in melanocytes, which inhibited melanoma formation in mice (356). In other cell types, such as prostate epithelial cells, IKKβ does not seem to play a role in tumor initiation, but is important in tumor progression under inflammatory circumstances (357). Recently IKKε, a member of the non-canonical pathway, has been identified as a breast cancer oncogene and functions to promote viral signaling pathways (358, 359). In contrast, IKKα has been reported as a tumor suppressor in head and neck, skin, and lung squamous cell carcinomas. Consistently, loss of IKKα expression cooperated with Ras to promote skin carcinogenesis (350, 360). Conditional knockout of the regulatory subunit IKKγ in hepatocytes induced spontaneous tumors in all mice, and also increased inflammatory cytokine expression (361). A similar phenotype was observed with conditional deletion of TAK1, a known activator of the IKK complex (362).

These studies report divergent functions of NF-κB family members in cancer development and progression, and indicate that the cell type plays an important role determining the oncogenic phenotype of NF-κB. Multiple factors could influence the outcome, including the stimulus involved in NF-κB activation. Along these lines, p65 may activate either pro- or anti-apoptotic genes (363). Campbell *et al.* (364) reported that

whereas TNF has been known to activate a p65-mediated anti-apoptotic gene program, ultraviolet light (UV) or daunorubicin/doxorubic led to the repression of an anti-apoptotic gene program. However, this same consequence was not observed upon doxorubicin treatment in other cell types, again indicating the cell-type specificities involved in NF-κB functions (365-367).

NF-κB in glioma. As with other cancers, NF-κB is constitutively active in glioma (368-371). In further support of an NF-κB prominent role in glioma, the gene encoding its inhibitor IκBα, *NFKBIA*, is commonly deleted in GBM (372), and its downregulation is associated with poor response to therapy (371). In a large study of 790 GBMs, an important relationship between loss of *NFKBIA* and EGFR was established, since *NFKBIA* deletion and *EGFR* amplifications were statistically mutually exclusive and had similar clinical outcomes (372). Introduction of *NFKBIA* in glioma cell lines led to sensitization to temozolomide, which appeared to be applicable to human tumors, since NFKBIA deletion has been shown to be a predictor for poor temozolomide response (371, 372). Further, *NFKBIA* expression and *MGMT* promoter methylation served as a 2 gene classifier to predict response to therapy (372). Another study examined human glioma tissue and observed a strong NF-κB correlation with Akt, implying a possible functional link in these pathways (348). Indeed, GBM cell lines demonstrated a requirement of Akt for NF-κB activation. Both NF-κB and Akt activation levels (phosphorylation) were associated with glioma grade in this study, providing further evidence that NF-κB is involved in glioma progression. Cell lines studies have demonstrated important roles for NF-κB in various tumor-promoting phenotypes. NF-κB was found to play a major role in driving the invasive phenotype in GBM (373), in part via IL-8 (369). As described in previous sections,

angiogenesis is a prominent hallmark of high-grade glioma. Inhibition of NF-κB in GBM cells led to decreased VEGF and IL-8 levels, accompanied by reduced angiogenesis in xenograft models (374). The investigations described above strongly suggest a potential therapeutic benefit of NF-κB inhibition in glioma, particularly in mediating chemosensitivity. NF-κB inhibition has been reported to sensitize glioma cells to both 5-aminolevulinic acid-based photodynamic therapy (5-ALA-PDT) and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (375, 376). Another report found that NF-κB activation was high in cisplatin-resistant C6 glioma cells. NF-κB inhibition reversed its activation and sensitized cells to both cisplatin and doxorubicin (377). NF-κB additionally appears to be important for maintaining glioma stem cells (378), since inhibiting this pathway induced glioma-initiating cells into senescence (379).

Therapeutic inhibition. NF-κB inhibition in cancer has been widely studied in pre-clinical models, as well as in the clinic. To date, no specific NF-κB inhibitors have been developed. Approaches for inhibition include general anti-inflammatory drugs, IKK inhibitors, and proteasome inhibitors. The broad anti-inflammatory inhibitors, including non-steroidal anti-inflammatory drugs (NSAIDs), inhibit NF-κB via an unknown mechanism, but have shown anti-tumor responses *in vitro* and in mouse models (380). Additionally, NSAIDs and natural compounds such as curcumin have been investigated as potential chemoprevention strategies (350). IKK inhibitors produce a more specific inhibition of the canonical NF-κB pathway. Due to animal models which demonstrated tumor inhibition in IKKβ knockouts, IKKβ antagonists have been developed and shown to be effective in multiple myeloma cells, large B-cell lymphoma, chronic myeloid leukimia, prostate cancer cells, and in melanoma xenografts (350). However, these inhibitors have yet to be studied

in the clinic, due the non-response in head and neck cancers and incomplete inhibition of NF-κB activity (381). This is likely due to the fact that NF-κB activity via the non-canonical pathway remains activated by IKKα and other kinases. Proteasome inhibitors, such as bortezomib, inhibit NF-κB activity by preventing degradation of IκBα inhibitor. Bortezomib has demonstrated positive outcomes in B-cell related cancers and is approved for therapy-resistant multiple myeloma (382). In contrast, it has shown little to no response in Hodgkin's lymphoma or solid cancers. Phase I studies are ongoing to assess its efficacy in combination with cytotoxic therapies.

Although NF-κB remains an attractive therapeutic target, caution should be taken when utilizing NF-κB inhibitors in the clinic, given the diverse outcome in mouse knockout studies and preclinical models. A recent report illustrates this concept by comparing the differential response of NF-κB inhibition in two separate studies (383-385). NF-κB inhibition was assessed in terms of mediating senescence-associated secretory phenotype (SASP) induced by cytotoxic therapy. The SASP response results in release of cytokines that arrest growth and signal the removal of senescent cells, and is therefore important for an effective anti-tumor effect (386, 387). These studies assessed the role of NF-κB inhibition on the SASP response. The outcome was based on the underlying genetic aberrations of the tumor. In the model proposed by Klein and Ghosh, a beneficial response to NF-κB inhibition would be produced in tumors with mutations in NF-κB pathway molecules, as the oncogenic function of NF-κB would be counteracted to promote cell death. On the other hand, in tumors where expression of survival molecules is independent of NF-κB (e.g., Bcl-2 expression), functional NF-κB is required to mediate therapy-induced senescence response by activating the transcription of genes of SASP (383). In the case, when a

genetic mutation is acquired independent of the NF-κB pathway, NF-κB inhibition could interfere with cytotoxic chemotherapy. Thus, context is crucial in predicting clinical outcome, and should carefully be considered as a stratification factor in future clinical trials.

Summary

IGFBP2 expression is correlated with glioma progression and is an independent predictor for survival. We have previously demonstrated that IGFBP2 directly promotes glioma progression in the RCAS/Ntv-a mouse model; however, the mechanism of action was unclear. We took the following observations into consideration: 1) We and others have demonstrated a functional invasive role of IGFBP2 binding to integrin $\alpha5\beta1$; 2) It is well documented that ILK is a major effector of integrin signaling and GFR signaling; and 3) ILK is known to activate NF-κB, and IGFBP2 overexpression in cell lines has led to NF-κB upregulation. Given these observations, **we hypothesized that IGFBP2 drives glioma progression through an integrin/ILK/NF-κB signaling pathway.**

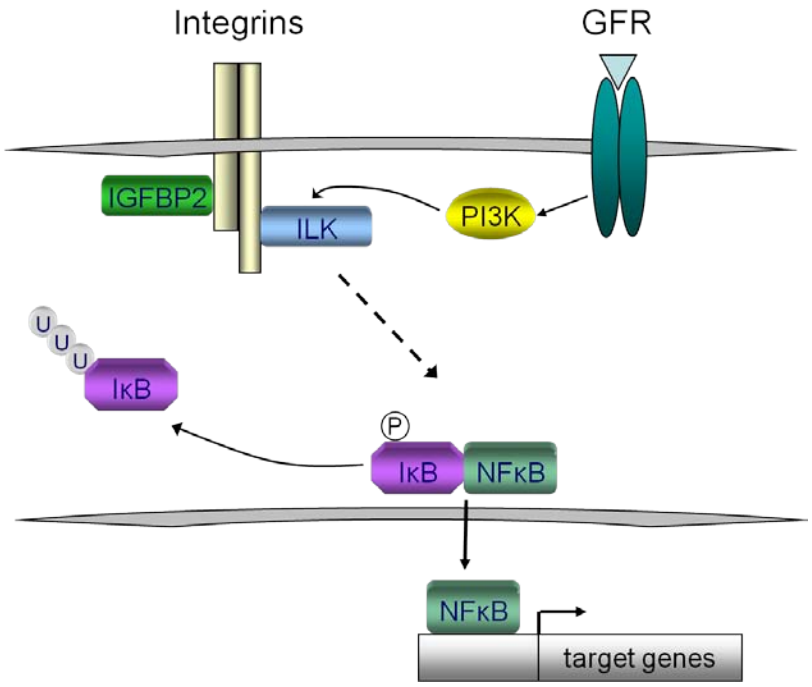


Figure 5. Proposed mechanism of IGFBP2-mediated glioma progression. IGFBP2 binding to integrin leads to recruitment of ILK and is activated in conjunction with GFRs, and in turn, leads to the activation of NF-κB which promotes glioma progression by activating cancer-promoting transcriptional programs.

CHAPTER 2: Materials and methods

Cell Culture and RCAS Constructs

SNB19 cells and IGFBP2 stably expressing SNB19 cells were maintained in Dulbecco's modified Eagle's medium/F-12 medium, supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic under 5% CO_2 at 37^{o}C. DF-1 cells were obtained from the American Type Culture Collection (ATCC) and were supplemented with 10% FBS and 1% antibiotic/antimycotic and grown under 10% CO_2 at 39^{o}C, according to ATCC instructions. RCAS constructs encoding the gene of interest were transfected into DF-1 cells via the calcium phosphate method. RCAS vectors encoding PDGFB and IGFBP2 have been previously described (86, 88). RCAS-IGFBP2(RGE) contains a D306E mutation and was subcloned into RCAS from the pcDNA3 vector. ILK cDNA was obtained from Upstate Biotechnology and subcloned into the RCAS vector. The ILK-KD contains a S343A mutation, which has been described previously (264). The RCAS vector containing IκBαM has S32,36A mutations and was purchased from Addgene. For the RCAS *ex vivo* infections, whole brains were taken from newborn Ntv-a mice, enzymatically dissociated, washed, and plated in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 1% antibiotic/antimycotic onto dishes under standard tissue culture conditions. Conditioned media from DF-1 cells transfected with the relevant RCAS construct were added overnight to the primary culture of glial progenitor cells, along with 5 ng/mL polybrene. Infections were repeated four times. Cells were treated with increasing nM amounts of PS-341 (gift from Dr. Paul Chiao, The University of Texas MD Anderson Cancer Center) for 24 hours.

Animal Care, RCAS Injection, and Tumor Pathology

DF-1 cells encoding RCAS viral particles were collected, mixed, and suspended in 1 x phosphate-buffered saline (PBS), and 2 μL of the cell suspension was subsequently injected into the right hemisphere of post-natal day 1 Ntv-a mice using a gas-tight Hamilton syringe. Mice were monitored daily for overall appearance and were euthanized at the first sign of distress (including hydrocephalus) or at the end of 13 weeks. The mice brains were removed and fixed in formalin for 24 hours, sectioned, and stained with hematoxylin and eosin. Tumors were graded by Greg Fuller, M.D., Ph.D. according to World Health Organization criteria. All animal experiments were performed in accordance with The University of Texas MD Anderson Cancer Center Institutional Animal Care and Use Committee.

Western Blotting

Cells were collected after treatment with trypsin, washed, and resuspended in RIPA buffer supplemented with protease and phosphatase inhibitors. Equal amounts of lysate were loaded onto a 10% polyacrylamide gel, resolved by SDS-PAGE, and transferred to a nitrocellulose membrane, followed by incubation with the appropriate antibody. Primary antibodies used to detect the appropriate antigens were as follows: phospho-integrin β1 (Thr788/789) (AB8123, Millipore), integrin β1 (4B7R) (ab3167, Abcam), phospho-FAK (Tyr397) (3283, Cell Signaling), FAK (3285, Cell Signaling), ILK (3862, Cell Signaling), GAPDH (sc-20357, Santa Cruz Biotechnology), p65 (sc-109, Santa Cruz Biotechnology), and p50 (sc-1190, Santa Cruz Biotechnology).

Immunofluorescence

For immunofluorescence analysis, 1 x 10^5 cells were seeded onto an uncoated glass slide cover-slip and cultured in the complete medium under the standard cell culture conditions. Cells were allowed to attach overnight and were then fixed in 4% paraformaldehyde at room temperature for 10 minutes, followed by permeabilization in 1 x PBS containing 0.5% Triton X-100 for 30 min at room temperature. The cells were then blocked in blocking solution (1 x PBS containing 10% normal goat serum and 0.05% Triton X-100) for at least 4 hr. After brief washing with 1 x PBS, the cells were incubated with a mouse monoclonal anti-human Rac1 antibody conjugated with FITC (#610652, BD Biosciences) (1:200 dilution in blocking solution) at 4^0C overnight. After washing, phalloidin staining was performed at room temperature for 45 min using phalloidin-TRITC (Molecular Probes, Invitrogen) at a concentration of 0.5 mg/ml. For the active form of integrin β1 staining, the cells were fixed in cold 100% methanol for 30 min at -20^0C and air dried for 15 min at room temperature. The integrin β1 staining was performed in the same manner as Rac1 staining, except a mouse anti-human integrin β1 (CD29) antibody (#556048, BD Biosciences) at 1:500 dilution was used. To visualize the integrin β1, a goat anti-mouse IgG conjugated with Alexa Fluor 488 (#A11029, Invitrogen) (1:1000) was incubated with the cells at room temperature for 1 hr. Images were captured by phase-contrast fluorescence microscopy (ZEISS HBO 100) at a magnification of 63x.

Flow Cytometry

Cells (5 x 10^5) were collected, washed in PBS, and transferred to a 5-mL fluorescence-activated cell sorting (FACS) tube, followed by fixation in 3.7% paraformaldehyde for 5 min. Cells were washed with PBS three times and incubated for 1

hr on ice with primary antibody at a 1:50 dilution in 1% bovine serum albumin (BSA)/PBS. Antibodies included were 12G10 (Ab30394, Abcam), HUTS-21 (556048, BD Pharmingen), and 4B7R (Ab3167, Abcam). Following incubation with primary antibody, cells were washed three times with PBS and incubated with a phycoerythrin (PE)-conjugated secondary antibody (goat α-mouse IgG-PE, sc-3738, Santa Cruz Biotechnology) at 1:500 in 1% BSA/PBS on ice for 30 min in the dark. Cells were washed three times with 1 x PBS containing 0.1% Tween20, resuspended in 1% BSA/PBS, and analyzed by FACS (FACSCalibur, BD Biosciences).

Bioinformatic analysis

Microarray experiments were carried out using the Human Whole Genome Oligo Microarray Kit from Agilent Technologies, and the manufacturer's protocol was followed (detailed protocol can be found on the Agilent website: www.Agilent.com). Briefly, 500 ng of total RNA from each sample was used and labeled with either Cy3- or Cy5-CTP. After 17 hr of hybridization at 65^0C, the arrays were washed and scanned with Agilent's dual-laser-based scanner. Then, Feature Extraction software GE2-v4_91 was used to link a feature to a design file and determine the relative fluorescence intensity between the two samples. Genes, along with their expression values, were uploaded to Ingenuity Pathway Analysis (IPA) v8.6-3003 (http://www.ingenuity.com) to perform pathway analysis. The differentially expressed genes were identified in IPA by screening out the genes with expression values below a twofold cutoff. The threshold was determined to ensure a sufficient number of genes eligible for network and pathway analysis, as required by the IPA software. The Ingenuity knowledge base (genes only) was used as a reference set. All data sources, all species, excluding uncategorized chemicals, and all tissues and cell lines were used for the

analysis. IPA uses computational algorithms to identify pathways that are particularly enriched for the input genes, with statistical significance as determined by Fisher's exact test.

We performed a human glioma analysis of gene expression microarray data from the NCI Rembrandt public data repository (http://rembrandt.nci.nih.gov) (388), which is composed of 329 tumors: 59 oligodendrogliomas, 102 astrocytomas, and 178 glioblastomas. Single gene survival analysis of human glioma was separated by midpoint expression value. Gene sets for all pathways were obtained from IPA software. The NF-κB target gene set was compiled from http://bioinfo.lifl.fr/NF-KB/ and TRANSFAC and composed of validated human genes. KEGG analysis was performed with the WebGestalt tool (http://bioinfo.vanderbilt.edu/webgestalt/option.php). Homo sapiens genome was used for all reference sets. A hypergeometric statistical method was employed with a Benjamini-Hochberg multiple test adjustment. The minimum number of genes per pathway was set at 2. For GSEA, gene sets were ranked according to Pearson correlation with IGFBP2, and an ES was calculated on the basis of the Komogorov-Smirnov statistic. P values were calculated by comparing ES to 1000 random permutations. Hierarchical clustering was performed on genes in integrin and ILK pathways that were correlated more than 0.5 (positive or negative). The distance metric was L1, using the unweighted mean distance as the linkage criterion. Groups were highlighted manually.

siRNA and Migration Assays

Stably expressing SNB19 cells were collected and plated onto six-well plates and subsequently transfected via RNAiMax (Invitrogen) with 50 nM siRNA directed against ILK or a scrambled negative siRNA control (Sigma). Cells were incubated to confluency for an additional 72 hr in 37^{o}C in a humidified chamber with 5% CO_2. A wound was made using a sterile 200-μL pipette tip, and cells were returned to the incubator. The ability of the cells to migrate into the wound area was assessed by photographing the wound area at 0, 6, 12, and 24 hr after the initial scratch was made. All photographs were taken from the same initial wound area. Each assay was done in triplicate. Cell migration was quantified using ImageJ (NIH software) to trace the wound area. The number of pixels inside the wound area was measured at each time point and compared to the initial wound area to calculate the percentage of wound closure.

Rac Activation Assay

The level of activated Rac was assessed in SNB19 cells stably expressing IGFBP2 using the Rac activation assay combo kit (STA-404, Cell Biolabs), according to the manufacturer's instructions. Cells were collected by manual cell scraping into 1 mL of lysis buffer with phosphatase and protease inhibitors 48 hours after siRNA transfections. Cell were allowed to lyse for 20 min and subsequently centrifuged to remove cellular debris. A portion of the supernatant (700μL) was added to 40μL of PAK-1 coated beads and were rotated for 1 hr at 4^{o}C. The beads were washed and loaded onto a 10% PAGE gel, transferred to a nitrocellulose membrane, and immunobloted for Rac1 (#610650, BD Biosciences). Levels of activated Rac were analyzed using ImageJ software by comparing

immunoblot band intensity between Rac from the pullodwn to total Rac in whole cell lysates.

EMSA

Cells were first incubated in a hypotonic buffer (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA) on ice for 20 min, NP40 was added to a final volume of 0.5% and briefly vortexed. Lysates were centrifuged at 14,000 rpm at 4^{o}C for 1 min to remove the cytoplasmic fraction. The nuclear pellet was washed in the hypotonic buffer, and nuclear extract buffer was added (20 mM Hepes, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 10% glycerol) and incubated for 15 min at 4^{o}C with constant vortexing. Finally, extracts were centrifuged at 14,000 rpm for 10 min at 4^{o}C. Five micrograms of nuclear extract was incubated with DNA binding buffer (100 mM Hepes, pH7.9, 400 mM NaCl, 10 mM EDTA, poly dI-dC, 10 mM DTT and ^{32}P-ATP-labeled NF-κB consensus probe, 40% glycerol) for 20 min at room temperature. Samples were resolved by a 5% polyacrylamide gel, transferred to filter paper, and detected via autoradiography. For supershift assays, the incubation was modified slightly by the addition of 1 µg of p65 (sc-7151X, Santa Cruz Biotechnology) or p50 (sc-114X) for 1 hr at 4^{o}C prior to the binding reaction.

Luciferase Reporter Assay

1.5 x 10^{5} SNB19 cells were plated onto 6-well plates and co-transfected 24 hours later with 500 ng of pGL4-NF-κB firefly luciferase and 20 ng of pGL4-TK Renilla luciferase constructs (Promega) via Lipofectamine 2000 (Invitrogen). Dual luciferase reporter assay system (Promega) was performed 48 hr after transfections, according to manufacturer's instructions, and luciferase activity was measured with a TD-20/20 luminometer. Each assay

was performed in triplicate, and all firefly luciferase values were normalized to renilla luciferase readings.

Statistical Analysis

Wound healing and luciferase assay results were analyzed by ANOVA, followed by Bonferroni's multiple comparison test. The log-rank test was used to obtain a p value for the significance of Kaplan-Meier curves' divergence. The statistical significance of the mouse tumor data was determined using a 2-ends Fisher's exact test. A significance level was set at $P < 0.05$ for all tests.

CHAPTER 3: Results

IGFBP2 is clinically linked to the integrin and ILK pathways

Rationale. IGFBP2 is highly expressed during fetal development and is downregulated following birth (112). However, similar to other developmental proteins, IGFBP2 levels are again increased during tumorigenesis (137), indicating that IGFBP2 likely is an important oncogene. Indeed, IGFBP2 is involved in many critical tumor-promoting processes, including migration (111), cell proliferation (147), invasion (161), angiogenesis (169), maintenance of glioma stem cells (172), and chemoresistance mechanisms (179). These phenotypes are also highly clinically applicable since high IGFBP2 expression levels correlate with poor prognosis in many cancers (137, 142, 149), and the increased serum levels in various malignancies has led to its proposal as a potential biomarker (154, 389).

Genomic studies allow for a bird's eye view of molecular events inside the cell and can be particularly useful in assessing the functional significance of gene perturbations. The application of cDNA microarrays following IGFBP2 overexpression has resulted in changes in adhesion, cellular migration and invasion, and cell proliferation (156, 390). We sought to obtain a clinically oriented global view of the IGFBP2 network by performing comprehensive pathway analyses using data from the Repository for Molecular Brain Neoplasia Data (Rembrandt) (388). This database was established by the National Cancer Institute as a public portal for correlation of molecularly characterized brain tumors with the respective clinical parameters. The Rembrandt database contains gliomas of all grades, providing a

unique opportunity to harness information on the molecular events involved in glioma progression. Pathway analysis was performed via IPA, which contains a curated list of genes reported to play a role in known signaling pathways.

Results. We obtained gene expression data from all glioma samples available in the Rembrandt database and determined which genes were correlated with IGFBP2. The gene list was uploaded IPA software to determine which pathways were associated with IGFBP2 expression (**Appendix Table A1**). Many of the top pathways (6 of 25) were related to cellular migration and invasion. Particularly, integrin and ILK were among the top associated pathways, and we chose to focus these pathways since IGFBP2 has been shown to bind integrin α5 and regulate cell motility through this interaction (134) and since ILK is a logical player in this pathway given its role in transducing integrin signaling. Notably, IGFBP2-correlated genes in the integrin pathway included multiple integrins: ITGA4, ITGB1, ITGA2, ITGA5, ITGA3, and ITGB3. The IGFBP2-correlated genes in the ILK pathway included MMP9, in accordance with IGFBP2-induced cellular invasion. Interestingly, an E-cadherin repressor, SNAI2, was altered among ILK pathway genes, indicating a potential EMT function for IGFBP2.

To determine whether integrin and ILK pathway genes were enriched in samples with high IGFBP2 expression, we performed a gene set enrichment analysis (GSEA). This method involves a pre-defined gene set (e.g., integrin pathway genes) and determines whether the gene set tends to occur at the top or bottom of the list (391). In this case, it would indicate whether the integrin pathway genes are significantly positively or negatively correlated with IGFBP2. Indeed, the genes in both the integrin and ILK pathways were significantly correlated with IGFBP2 ($P<0.001$; **Figure 6 and Appendix Table A2**).

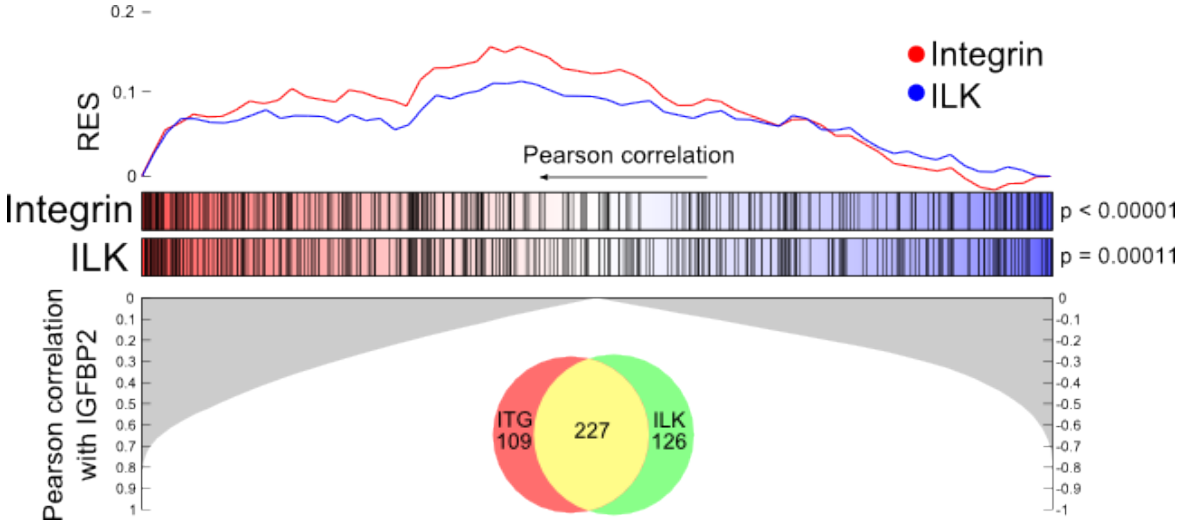


Figure 6. IGFBP2 is associated with integrin and ILK pathways in human glioma. This figure shows GSEA for integrin and ILK pathway genes, based on their correlation with IGFBP2 expression. Maximum running enrichment score (RES) for integrin and ILK pathway genes are 0.1602 and 0.1182, respectively. Venn diagram represents the number of pathway genes that are unique, or shared between integrin and ILK.

To determine the expression patterns of integrin and ILK pathway genes in accordance with IGFBP2 expression and glioma grade, we performed unsupervised hierarchical clustering. Three major clusters formed, including low, intermediate, and high expression (**Figures 7 and 8**). The clusters were related to IGFBP2 expression and tumor grade, with the high expression cluster also containing the highest IGFBP2 expression and mostly glioblastoma, indicating a possible positive regulation of integrin and ILK pathway genes by IGFBP2. The pathway genes that were not correlated with IGFBP2 expression (bottom rows in **Figures 7 and 8**) clearly demonstrated an opposing pattern with IGFBP2, suggesting a possible negative regulation.

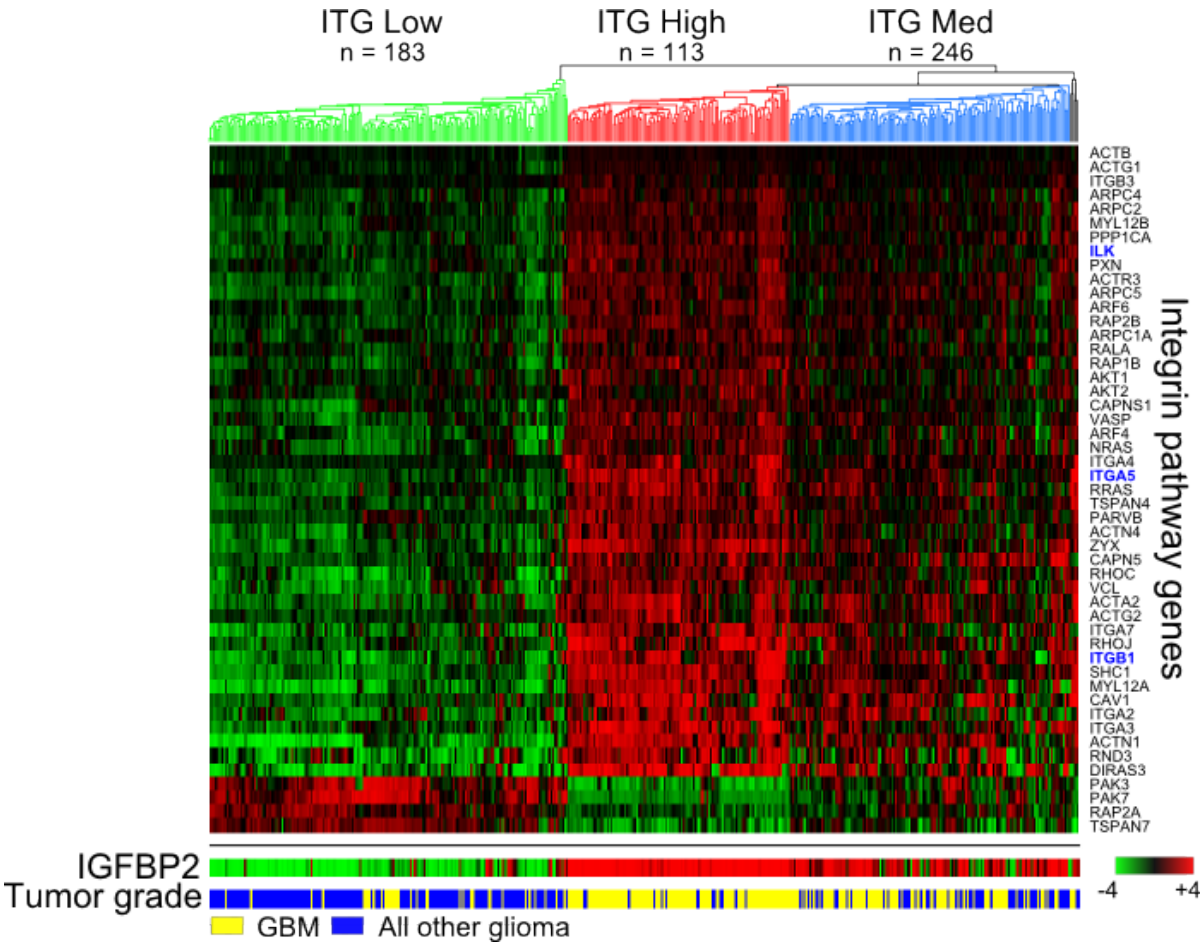


Figure 7. Integrin pathway genes cluster according to IGFBP2 and tumor grade. Hierarchical clustering of integrin pathway genes that are correlated (greater than 0.5 Pearson correlation) with IGFBP2. Specific molecules assessed in this study are highlighted in blue (ILK, ITGA5, and ITGB1).

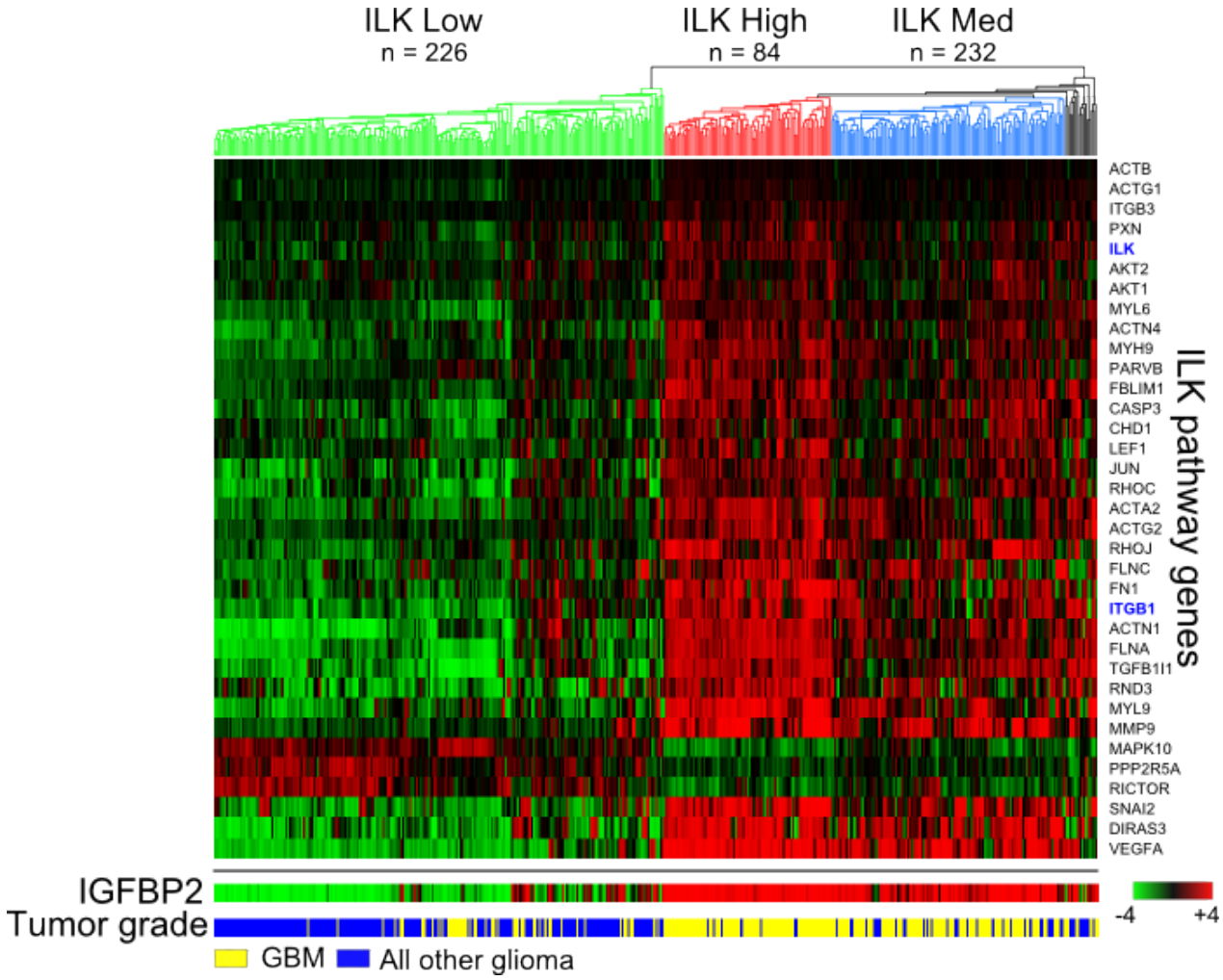


Figure 8. ILK pathway genes cluster according to IGFBP2 and tumor grade.
Hierarchical clustering of ILK pathway genes that are correlated (greater than 0.5 Pearson correlation) with IGFBP2. Specific molecules assessed in this study are highlighted in blue (ILK and ITGB1).

To determine the influence of integrin and ILK pathway gene expression on patient survival, we assessed individual genes, including *IGFBP2*, *ITGA5*, *ITGB1*, and *ILK*, among all tumor histologies (GBM, Astrocytoma, Oligodendroglioma, Mixed). “High” and “Low” groups were separated by the midpoint expression value. High expression of each gene was associated with significantly poorer survival (**Figure 9**). This survival trend remained upon individual evaluation of each glioma histology (**Appendix Figure A1**). In addition, expression levels of integrin and ILK pathway genes (low-, intermediate-, and high-expression clusters from Figures 7 and 8) were significantly correlated with patient survival, highlighting the clinical significance of the integrin and ILK pathways in human glioma (**Figure 9**).

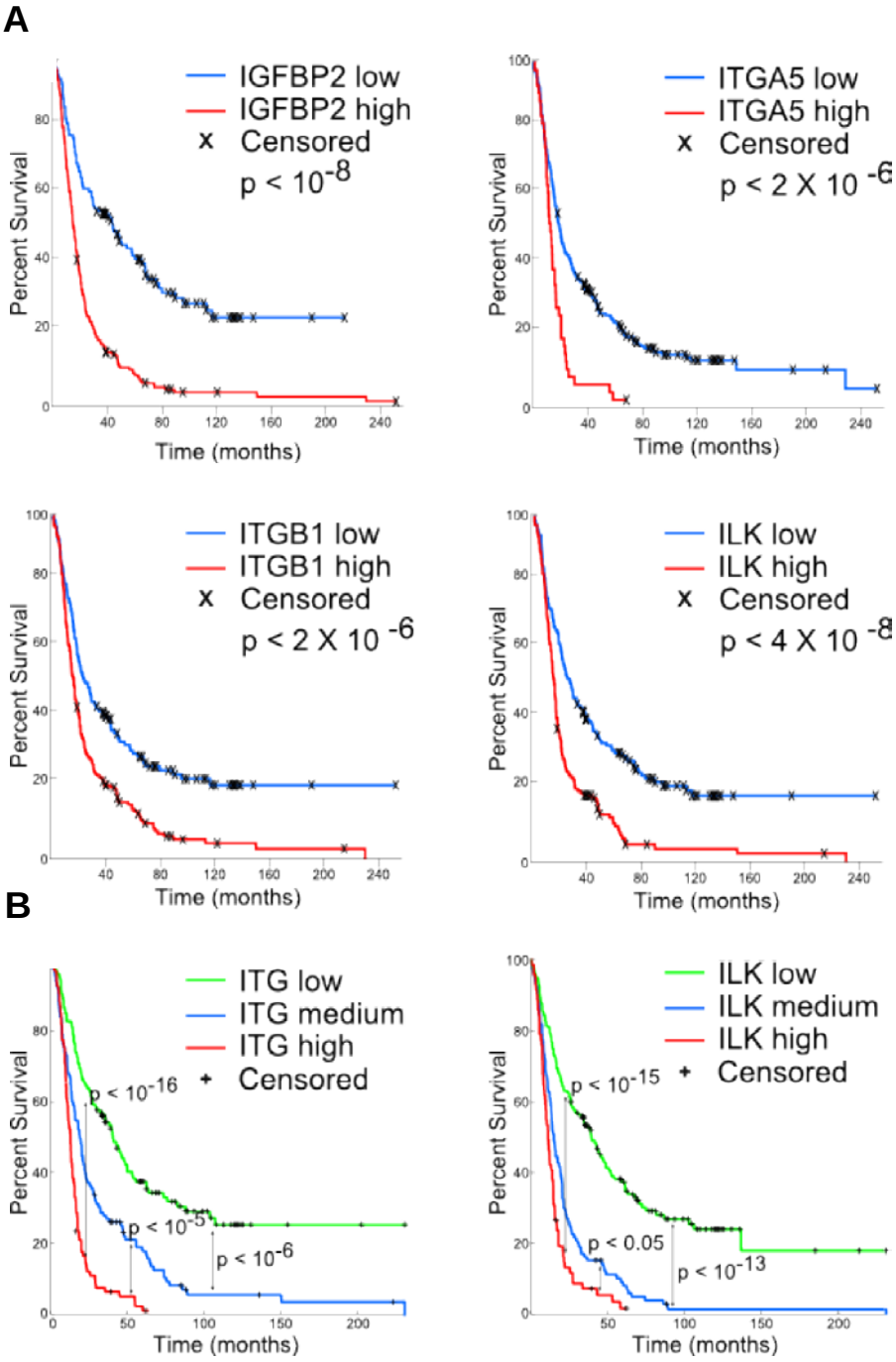


Figure 9. High expression of IGFBP2-related genes is associated with poor prognosis. (A) Single-gene Kaplan-Meier survival curves of all glioma samples (n=329). P values were calculated using the log-rank test. (B) Kaplan-Meier survival curves based on clusters in Figures (X) and (Y). P values were calculated using the log-rank test.

IGFBP2 regulates downstream pathways via integrin activation

Rationale. IGFBP2 has been reported to interact with α5β1 (133, 134) as well as αvβ3 (136); however in our previous study, IGFBP2 did not immunoprecipitate αv (134). Further, while addition of anti-α5β1 could decrease IGFBP2 binding to cell surfaces, anti-αvβ3 antibodies had no effect (133). Although IGFBP2 likely influences other integrins, we focused on integrin β1 to investigate integrin pathway, given that integrin β1 comprises the most heterodimers and is one of the better known oncogenic integrins involved in cell migration, invasion, and metastasis.

Although the IGFBP2/integrin α5β1 interaction was documented, it was unknown whether this interaction could induce integrin activation. Integrin clustering, formation of focal adhesions, and subsequent cell signaling transduction are all byproducts of activated integrins (185); therefore, detection of integrin activation is important in determining functional significance. As described previously, activated integrins shift from a bent conformation with a closed headpiece to an upright conformation, including separation between the α and β transmembrane portions and cytoplasmic tails (206). Conformation-specific antibodies have been developed which bind to epitopes exposed only when the integrin is in the active conformation. We utilized two separate conformation-specific antibodies, both of which detect activated β1 integrin: 12G10 binds to an epitope in the βA domain, and HUTS-21 binds an epitope in the hybrid domain (392, 393). An antibody that recognizes all integrin β1 conformations, 4B7R, was used as a control.

To address whether IGFBP2 activates integrin β1 and its downstream pathways we compared 2 stably expressing cell lines originating from SNB19: 2 clones expressing a mutant form of IGFBP2 that cannot bind integrin (RGD→RGE point mutation; referred to as RGE-mutant) and 2 clones expressing wild-type IGFBP2.

Results. We performed immunofluorescence analysis and flow cytometry experiments to assess whether IGFBP2 activates integrin β1. Levels of activated β1 were compared among SNB19 parental cells; cells stably expressing IGFBP2; and cells stably expressing RGE-mutant IGFBP2. Both parental and RGE-mutant cells exhibited a moderate level of activated β1 staining. Activated integrins were localized primarily on focal adhesions, indicating the cells were non-motile and engaged with the ECM. In contrast, IGFBP2-expressing cells had high levels of staining, localized primarily along the leading edge of the cell, indicating their motile nature (**Figure 10A**). We used an additional approach to further confirm that IGFBP2 activates integrin β1 by performing flow cytometry. These results mirrored the immunofluorescence data. The parental and RGE-mutant cells exhibited low levels of activated integrin, as measured with the HUTS-21 or 12G10 antibodies, whereas there was a shifted peak in IGFBP2-overexpressing cells, indicating that a higher proportion of cells was recognized by these antibodies (**Figure 10B**). 4B7R again served as the control, with similar levels of total β1 integrin among the cell lines, indicating that the difference in β1 activation was not merely due to increased expression.

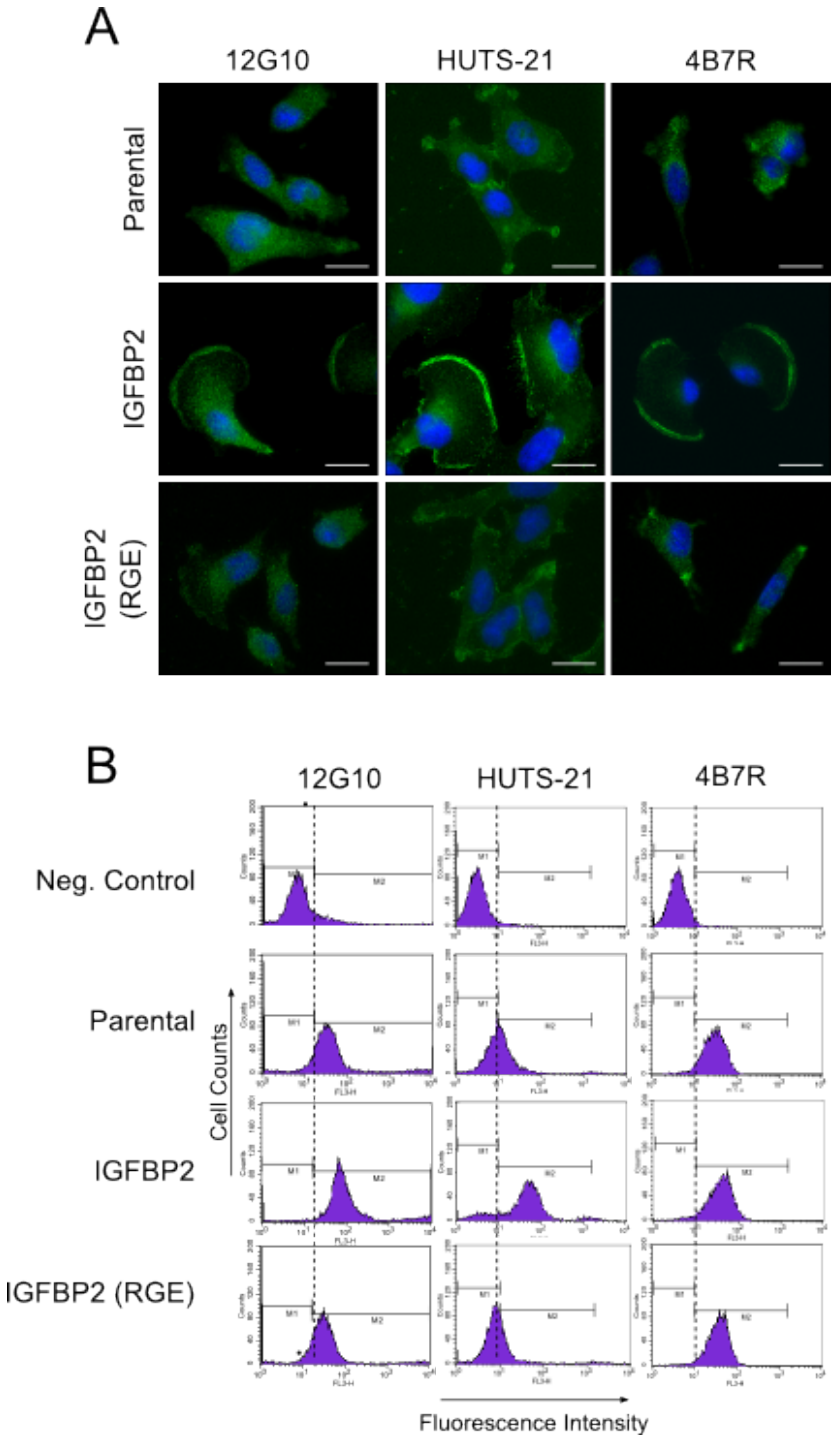


Figure 10. IGFBP2 activates integrin β1. (A) Immunofluorescence staining of 12G10, HUTS-21, and 4B7R in indicated cells after plating onto fibronectin-coated glass coverslips for 18 hr, followed by cold-methanol fixation. Scale bar, 20 μm. (B) Flow cytometric analysis of 12G10 and HUTS-21 (active conformation of β1) or 4B7R (total β1) in the indicated cells.

To determine the significance of the downstream pathways affected by disruption of IGFBP2/integrin signaling, we performed a cDNA microarray analysis comparing SNB19 RGE-mutant and IGFBP2 wild-type cells. Differentially expressed genes were subjected to IPA. Disruption of integrin binding with IGFBP2 (RGE-mutant) led to many significantly altered pathways (**Appendix Table A3**). The integrin pathway was confirmed to be altered, and the third top altered pathway was ILK, followed by other pathways involved in migration and invasion. GSEA was performed to determine if genes in the selected pathways were significantly enriched according to IGFBP2 expression. GSEA may be measured based on either differential expression or absolute differential expression. When differential expression is used, GSEA emphasizes gene sets in which all of the genes go either up or down. Absolute differential expression does not take into account the direction of change, but determines only if there is a significant change in gene expression. We performed GSEA was based on both differential and absolute differential gene expression using four selected Ingenuity pathways, including integrin, ILK, actin cytoskeleton, and glioma invasiveness. The absolute differential expression GSEA revealed that each pathway was significantly enriched, and the differential expression GSEA showed that most genes were down-regulated, indicating that disruption of IGFBP2 and integrin binding led to de-activation of cell motility pathways (**Figures 11-14**).

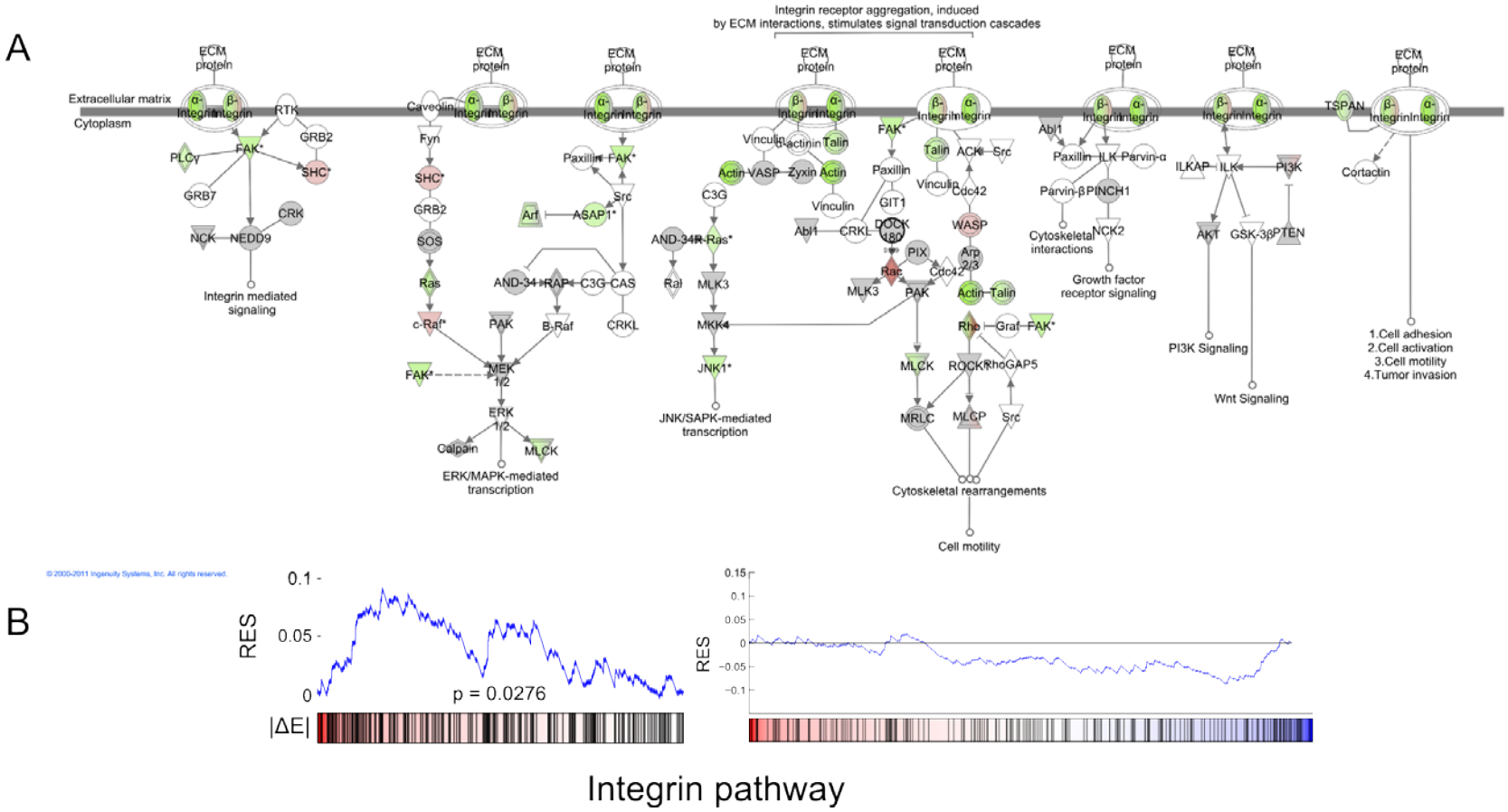


Figure 11. Disruption of IGFBP2-integrin interaction alters genes in the integrin pathway. (A) The integrin pathway is depicted from Ingenuity pathway analysis showing gene expression changes from SNB19 glioma cells comparing clones expressing wild-type IGFBP2 to RGE-mutant IGFBP2. Green and red represent genes represent expression fold changes >2 or <2, respectively. (B) Integrin pathway genes are significantly enriched according to GSEA analysis. Most genes were negatively affected, revealing decreased activity of the integrin pathway.

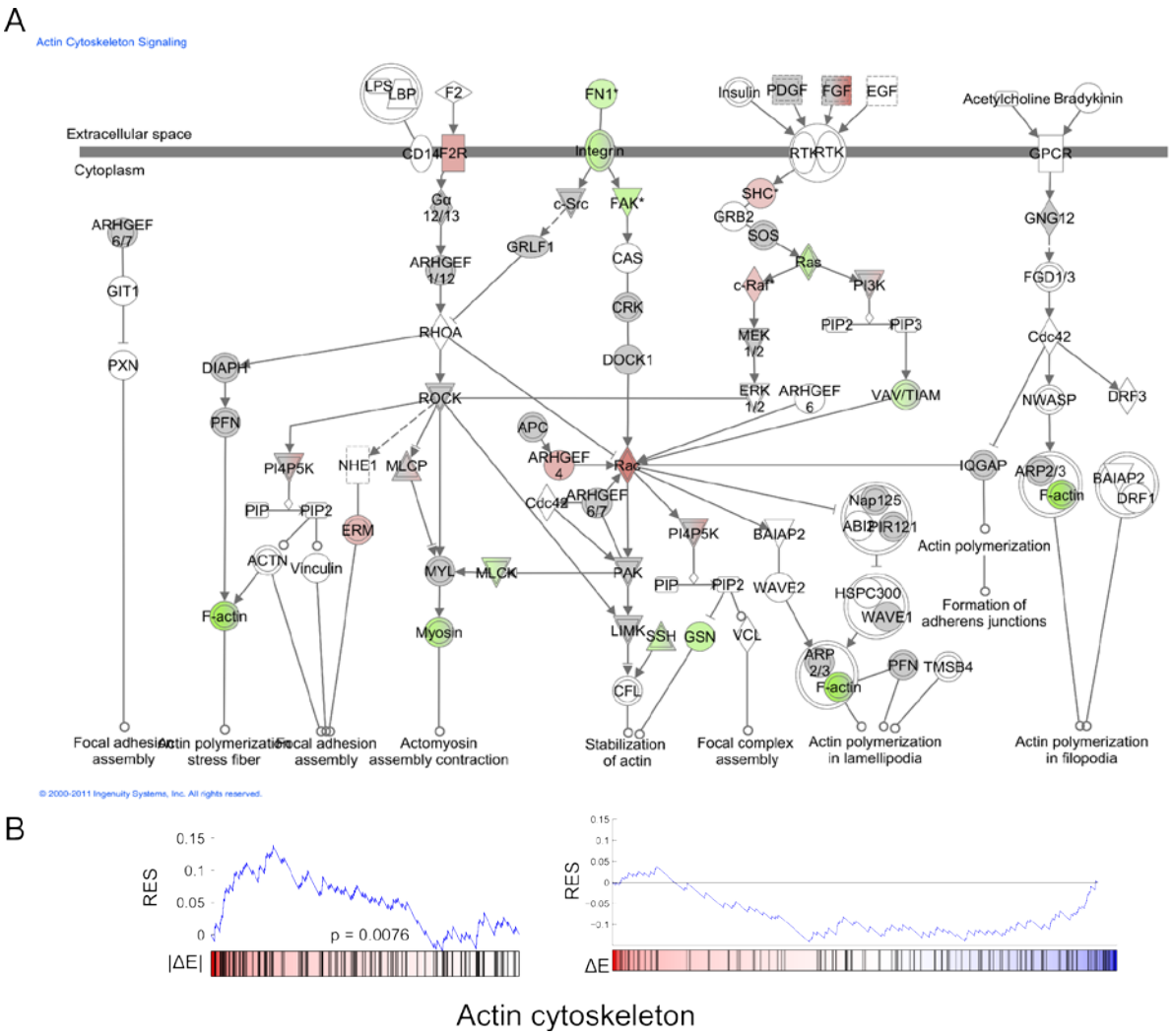


Figure 12. Disruption of IGFBP2-integrin interaction alters the actin cytoskeleton pathway. (A) The actin cytoskeleton pathway is depicted from Ingenuity pathway analysis showing gene expression changes from SNB19 glioma cells comparing clones expressing wild-type IGFBP2 to RGE-mutant IGFBP2. Green and red represent genes represent expression fold changes >2 or <2, respectively. (B) Actin cytoskeleton pathway genes are significantly enriched according to GSEA analysis. Most genes were negatively affected, revealing decreased activity of the actin cytoskeleton pathway.

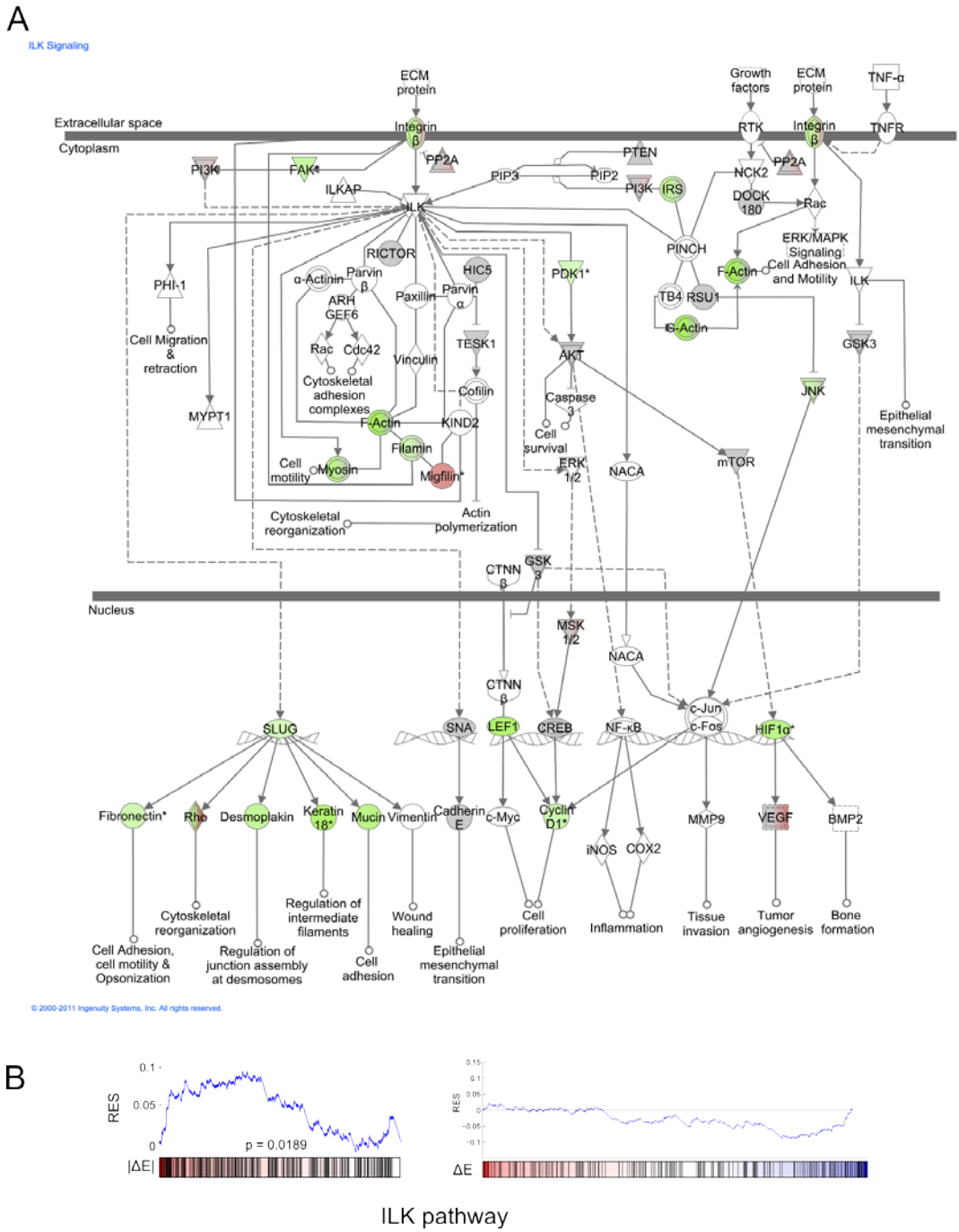


Figure 13. Disruption of IGFBP2-integrin interaction alters the ILK pathway. (A) The ILK pathway is depicted from Ingenuity pathway analysis showing gene expression changes from SNB19 glioma cells comparing clones expressing wild-type IGFBP2 to RGE-mutant IGFBP2. Green and red represent genes represent expression fold changes >2 or <2, respectively. (B) ILK pathway genes are significantly enriched according to GSEA analysis. Most genes were negatively affected, revealing decreased activity of ILK signaling.

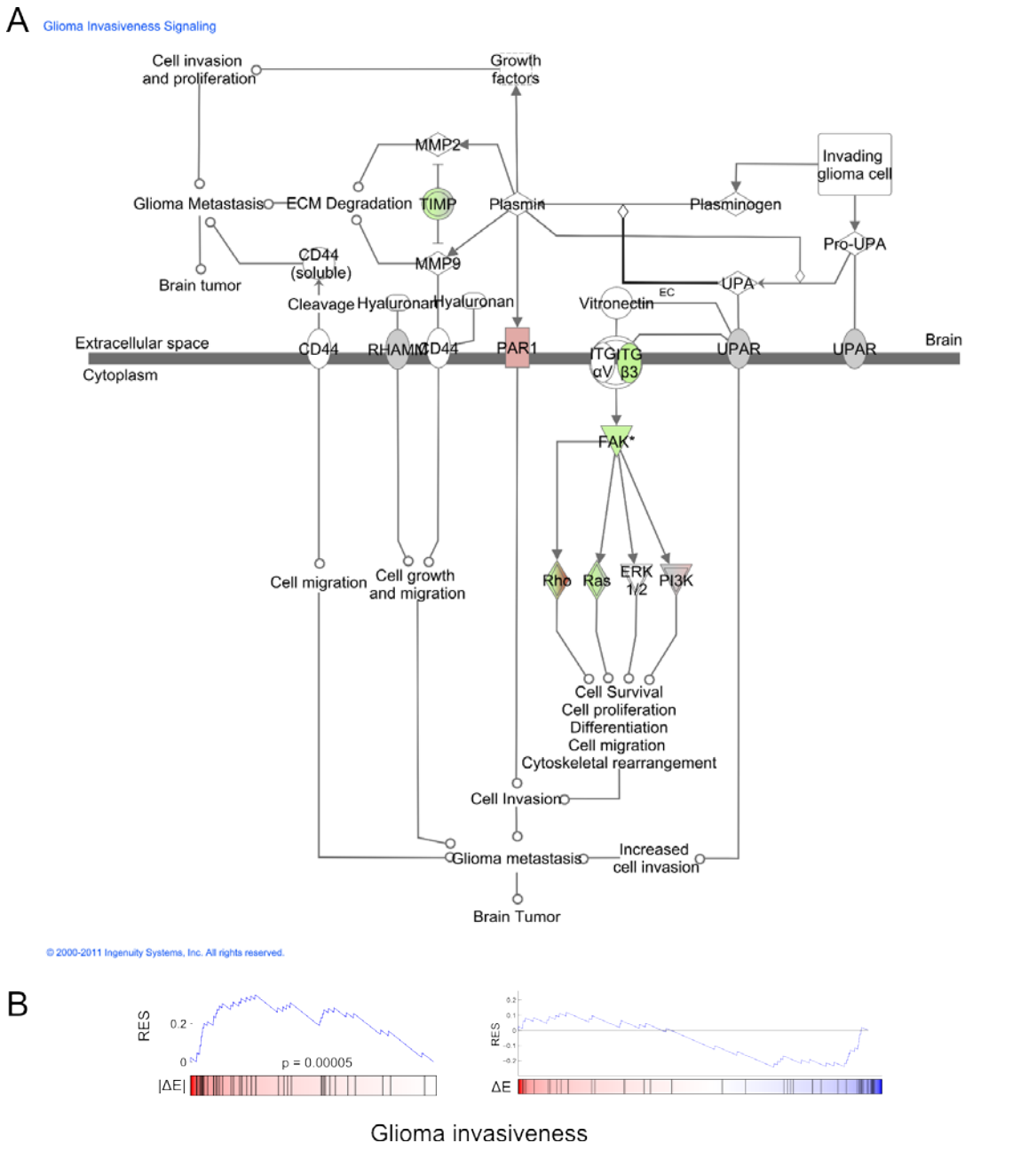


Figure 14. Disruption of IGFBP2-integrin interaction alters the glioma invasiveness pathway. (A) The glioma invasiveness signaling pathway is depicted from Ingenuity pathway analysis showing gene expression changes from SNB19 glioma cells comparing clones expressing wild-type IGFBP2 to RGE-mutant IGFBP2. Green and red represent genes represent expression fold changes >2 or <2, respectively. (B) Glioma invasiveness pathway genes are significantly enriched according to GSEA analysis. Most genes were negatively affected.

ILK is required for IGFBP2-induced cell mobility

Rationale. ILK binds the cytoplasmic tails of integrins β1 and β3, and is an essential effector of integrin and growth factor signaling (265). As such, ILK regulates cellular migration and invasion, proliferation, angiogenesis, EMT, and other functions that cancer greatly relies upon. Multiple lines of evidence have led us to the hypothesis that ILK plays a critical role in IGFBP2-mediated effects. Integrin clustering is known to recruit ILK to transduce appropriate signaling. Since IGFBP2 activates integrin β1, ILK is likely recruited to the complex. We demonstrated in human glioma samples that ILK pathway genes were significantly correlated with IGFBP2 and clustered according to IGFBP2 expression. Additionally, perturbation of IGFBP2/integrin binding negatively affected genes in the ILK pathway.

We previously reported that IGFBP2 promotes migration through integrin interaction and activation of Rac, a small GTP-binding protein critical for lamellipodia and subsequent cell motility (134). We sought to determine whether ILK was critical for cell motility and Rac activation induced by IGFBP2. To address this question, we used an siRNA approach to knockdown ILK in SNB19 cells stably overexpressing IGFBP2. Wound healing assays were performed to assess cell migratory abilities. The assay entails creating a scratch in confluent cells and monitoring the wound area over time. The extent of cell migration is reflected by the wound closure rate. Rac activation was assessed by immunofluorescence of filamentous actin (F-actin) and Rac, since a crucial Rac function involves polymerization of actin monomers into F-actin which is necessary for cell tension and movement. The Rac activation assay is a pulldown approach based upon the premise that the active GTP-bound Rac interacts with PAK.

Results. ILK knockdown resulted in a marked decrease in migration in IGFBP2-expressing cells compared with control siRNA or parental cells (**Figure 15A**). IGFBP2-expressing SNB19 cells migrated into the wound in a significantly higher proportion than did parental cells ($p<0.01$; **Figure 15B**). In contrast, IGFBP2 cells treated with ILK siRNA exhibited a significant decrease in the wound closure rate relative to IGFBP2 cells treated with control siRNA ($p<0.05$; **Figure 15B**), indicating that ILK is an essential component of IGFBP2-induced cell migration. A Western blot analysis confirmed that ILK was sufficiently knocked down and further revealed that ILK protein levels were increased in IGFBP2-overexpressing cells (**Figure 15C**).

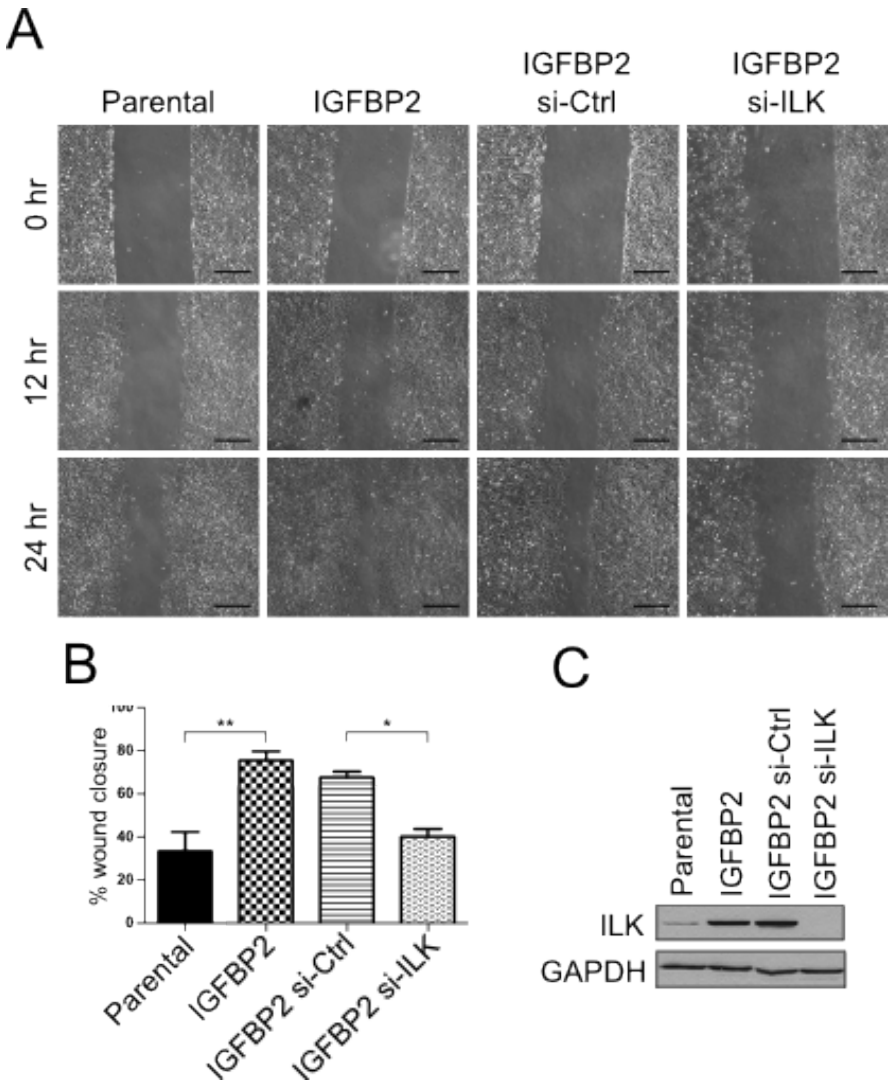


Figure 15. ILK is required for IGFBP2-induced cell motility. (*A*) Wound-healing assay in SNB19 parental or IGFBP2-expressing cells transfected with negative control or ILK siRNA. Images were captured at the indicated times after the initial wound. Scale bar, 500 μm. (*B*) Graphical representation of wound healing ability after 24 hr, as shown in (*A*). P values represent ANOVA with Bonferroni's multiple comparison test. *p<0.05, **p<0.01. Error bars represent SEM. (*C*) Western blot analysis of ILK protein levels in SNB19 cell lines.

Since we previously reported that IGFBP2/integrin binding led to activation of Rac (134), we determined whether ILK is also critical in this pathway. Immunofluorescence staining of F-actin and Rac demonstrated that F-actin was localized mostly to focal adhesions or throughout the cell membrane in parental cells but concentrated on the lamellipodia in IGFBP2 cells. Upon ILK knockdown, the cells' morphologic characteristics shifted to a non-motile phenotype by becoming elongated with fewer lamellipodia, and F-actin was present mainly in the focal adhesions. Rac was dispersed throughout the cytoplasm in parental cells but localized at the leading edge with F-actin in IGFBP2 cells, indicating Rac activation. Knockdown of ILK in IGFBP2 cells led to Rac's movement to the cell tail (**Figure 16A**). To confirm Rac activation in these cells, we performed a Rac activation assay. IGFBP2 cells with depleted ILK exhibited significantly less activated Rac than did IGFBP2 cells that were untreated or treated with control siRNA (**Figures 16A and B**). These results indicate that ILK plays a direct role in IGFBP2/integrin α5-induced glioma cell migration.

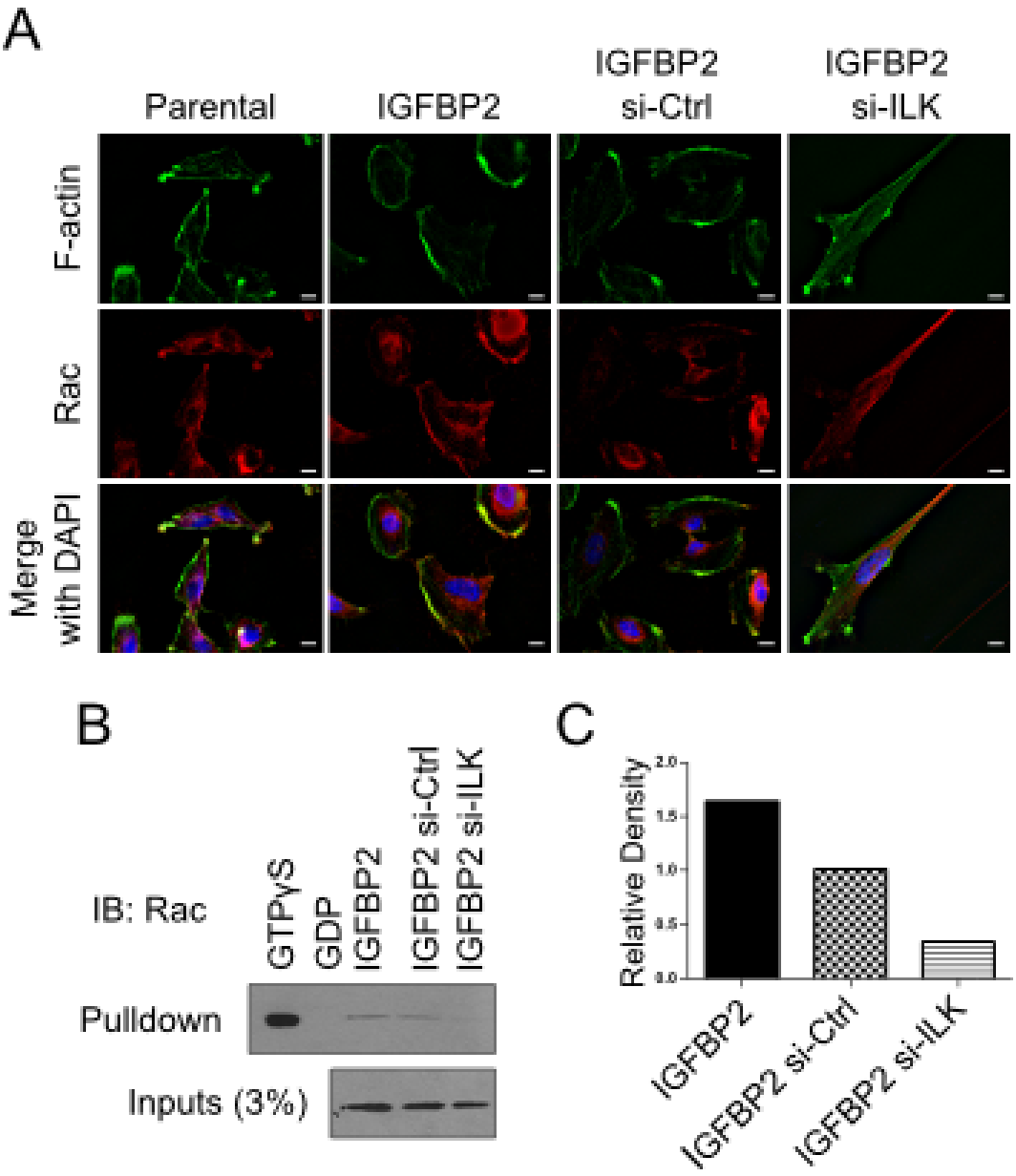


Figure 16. ILK is required for IGFBP2-induced Rac activation. (A) IGFBP2-expressing SNB19 cells transfected with negative control or ILK siRNA for 48 hr, followed by cold methanol fixation and immunofluorescence staining of F-actin and Rac. (B,C) Rac activation assay in IGFBP2 cells that were untreated or transfected with scrambled or ILK siRNA. (C) Quantification of the amount of immunoprecipitated Rac1 normalized to the amount of Rac1 in the inputs.

IGFBP2 regulates an invasion-related NF-κB transcriptional program

Rationale. NF-κB is one of the most influential transcription factors in cancer, as it has been documented to regulate over 150 genes, many of which perform cancer-promoting functions (327, 394). Similar to IGFBP2, NF-κB is constitutively active in glioma and correlates with tumor grade (348). IGFBP2 has been reported to upregulate NF-κB in a breast cancer cell line (390), and a previous study by our group revealed that inhibition of IGFBP2-induced cell migration via IIp45 (also known as MIIP) led to decreased NF-κB expression (163). Additionally, IGFBP2 regulates integrin and ILK signaling, which are both known activators of NF-κB (395-397). These observations led us to hypothesize that IGFBP2 mediates oncogenic functions through NF-κB.

We compiled validated human NF-κB target genes and utilized the Rembrandt database to assess whether IGFBP2 expression correlated with the expression of NF-κB target genes. To determine whether NF-κB was activated, we first assessed its localization by performing nuclear subfractionation and analyzing levels of nuclear NF-κB. Because nuclear localization is merely indicative of activation, we also determined the ability of NF-κB to bind its consensus sequence via electrophoretic mobility shift assays (EMSA), and its transactivation potential by performing luciferase gene reporter assays. Finally, we assessed whether IGFBP2 expression rendered cells more sensitive to NF-κB inhibition via the proteasome inhibitor PS-341 (bortezomib) or to the dominant negative genetic inhibitor, IκBαM. Since we had previously found that IGFBP2 (in combination with PDGFB) induced glioma progression in a spontaneous mouse model (88), we utilized this system for *ex vivo* NF-κB inhibitor experiments.

Results. We evaluated NF-κB target gene expression from both the SNB19 cell line microarray and Rembrandt database. GSEA based upon absolute differential expression (**Fig. 17, top panel**) revealed that NF-κB target genes were significantly enriched in stably expressing IGFBP2 cell lines. GSEA based upon differential expression (**Fig. 17, bottom panel, Table 1**) revealed both upregulation and downregulation of NF-κB target genes, consistent with the diverse functions of NF-κB target genes. These results provide evidence that IGFBP2 regulates NF-κB activity.

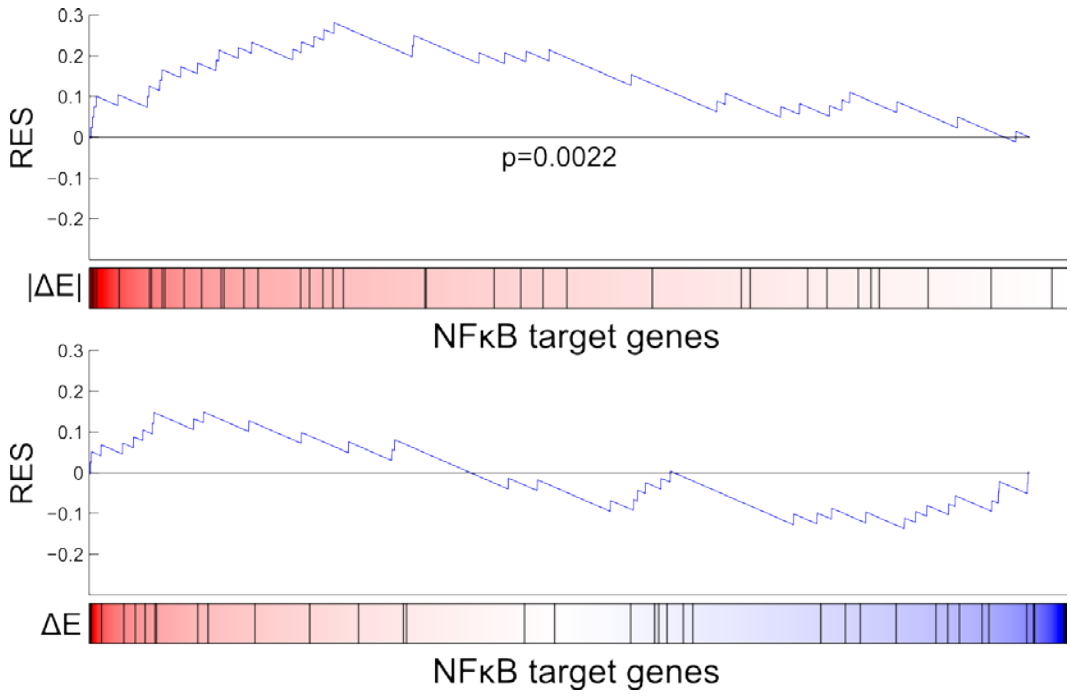


Figure 17. Disruption of IGFBP2/integrin significantly alters NF-κB transcriptional activity. GSEA plot of NF-κB target genes. Genes ranked according to differential expression of SNB19 stably expressing IGFBP2 cells to control cells.

NF-κB target gene	Expression fold change
IL11	2.58
F3	2.29
PLAU	1.40
PTX3	0.94
TNC	0.83
BCL2L1	0.77
CCND1	0.72
CD83	0.71
CREB3	0.54
MUC5B	0.51
NFKB1	0.41
NFKBIA	0.31
MICA	0.23
COL1A2	0.17
CD44	0.17
BCL2A1	0.04
HMOX1	0.01
PLCD1	-0.07
IL6	-0.10
MYC	-0.10
GSTP1	-0.11
HIF1A	-0.13
SOD2	-0.14
TPMT	-0.29
NQO1	-0.33
ING2	-0.35
AR	-0.41
TNFRSF10B	-0.50
FAS	-0.52
IL15	-0.56
RELB	-0.63
S100A6	-0.66
TAP1	-0.93
BCL3	-1.01
HLA-G	-1.03
CCL2	-2.95
SPP1	-3.33

Table 1. Differentially expressed NF-κB target genes resulting from IGFBP2 overexpression.

The Kolmogorov-Smirnov statistic was used to determine whether NF-κB target genes were significantly correlated with IGFBP2 expression in human glioma. NF-κB target genes were significantly enriched ($P<0.01$; **Figure 18A and Table 2**), although not as robustly as in the integrin and ILK pathways. Since NF-κB regulates many genes, resulting in pleiotropic effects ranging from immune regulation to cellular invasion, we therefore evaluated the NF-κB target genes that were positively correlated with IGFBP2. A Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis revealed that these genes were enriched in migration and invasion-related pathways, such as extracellular matrix-receptor interaction and focal adhesion. In contrast, target genes that were not correlated with IGFBP2 (Pearson correlation below 0.5) were not enriched in migration and invasion pathways, indicating that IGFBP2 likely activates an invasion-related NF-κB transcriptional program (**Figure 18B**).

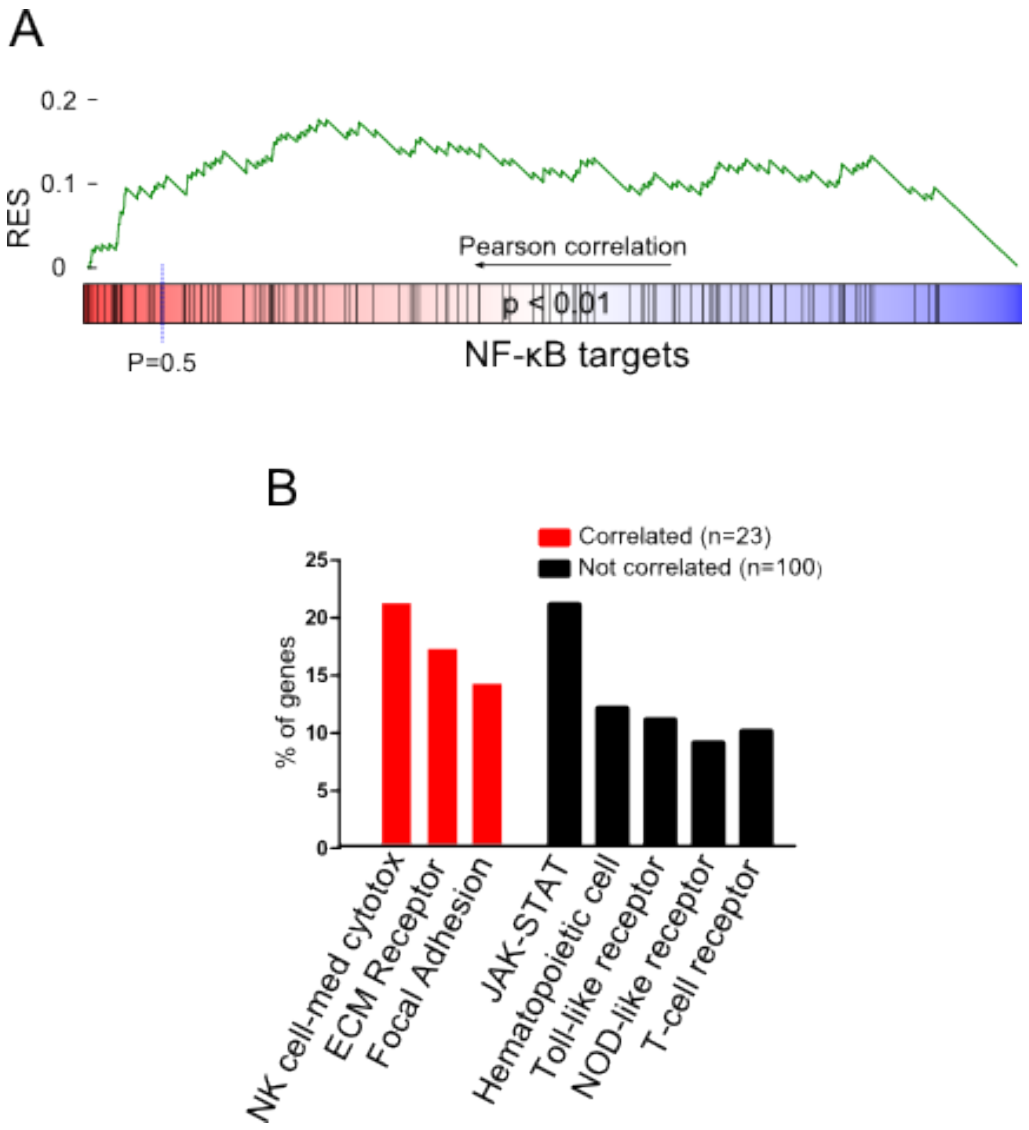


Figure 18. IGFPB2 activates an NF-κB transcriptional program. (A) GSEA plot from human glioma Rembrandt database. NF-κB target genes ranked according to IGFBP2 correlation. (B) KEGG analysis of genes from (A). Pathways shown are significantly enriched in NF-κB target genes that are correlated >0.5 (red bars) or <0.5 (black bars) with IGFBP2. Percentage of genes represents the number of NF-κB target genes in the pathway relative to the total number of correlated or non-correlated genes.

NF-κB target gene	Pearson correlation	Gene	Pearson correlation	Gene	Pearson correlation
COL1A2	0.767	TGM2	0.284	CD209	-0.116
PTX3	0.749	HIF1A	0.279	LTB	-0.126
IFNGR2	0.737	IL15	0.272	NR4A1	-0.126
SOD2	0.708	NOD2	0.268	CRP	-0.132
PLAU	0.671	CCR5	0.258	AGER	-0.135
S100A6	0.643	CXCL9	0.256	GUCY1B3	-0.138
TNFRSF10B	0.617	IL1RN	0.245	NR4A2	-0.139
F3	0.612	PTGS2	0.22	IFNB1	-0.141
BCL3	0.611	IRF2	0.214	SCNN1A	-0.15
MMP9	0.609	NFKB2	0.205	IL13	-0.158
HMOX1	0.606	VCAM1	0.204	TPMT	-0.162
BRCA2	0.605	IL10	0.185	ELF3	-0.164
TNC	0.597	REL	0.156	TACR1	-0.175
HLA-G	0.595	CSF1	0.145	BDKRB1	-0.185
SPP1	0.594	OLR1	0.141	CCL11	-0.185
ALOX5AP	0.591	CXCL5	0.14	TAC1	-0.197
IRF1	0.559	IL1A	0.135	CD40LG	-0.211
CD44	0.555	ING2	0.117	NPY1R	-0.217
TNFAIP3	0.529	IL1B	0.104	MUC5B	-0.22
TAP1	0.526	IL2RA	0.104	IL9	-0.236
IL8	0.519	CREB3	0.09	FASLG	-0.244
FAS	0.505	PDGFB	0.08	IL2	-0.246
ICAM1	0.501	AR	0.069	STAT4	-0.247
TP53	0.458	PTAFR	0.069	IRF4	-0.248
CCL2	0.457	CD80	0.046	TNF	-0.26
IRF7	0.457	SELP	0.039	IL5	-0.264
RELB	0.448	TNFRSF9	0.018	OPRM1	-0.27
MICA	0.439	CCND1	0.007	IL29	-0.277
BCL2A1	0.425	SELE	0.007	LTA	-0.281
CD74	0.425	CCR7	0.002	ALOX12B	-0.333
NFKB1	0.412	CR2	-0.008	CCL19	-0.358
SAA1	0.406	CD83	-0.019	CRMP1	-0.37
STAT5A	0.397	APOC3	-0.022	ABCB1	-0.373
HAS2	0.393	MYC	-0.024		
BCL2L1	0.355	IFNG	-0.025		
IL6	0.355	IGF1	-0.031		
GSTP1	0.34	IL11	-0.035		
NOS2	0.334	CD3G	-0.067		
NQO1	0.328	PLCD1	-0.076		
TFPI2	0.319	CSF3	-0.078		
SLC2A5	0.318	IRF8	-0.082		
GATA3	0.317	IL12B	-0.085		
CCL5	0.312	NFKBIA	-0.087		
CD48	0.307	OPRD1	-0.101		
IL15RA	0.299	CSF2	-0.105		

Table 2. Pearson correlation of NF-κB target genes with IGFBP2 in human glioma.

To validate that IGFBP2 activates NF-κB and to determine the role of integrin signaling in NF-κB activation, we used SNB19 parental, RGE-mutant, and wild-type IGFBP2 cells. A Western blot analysis of the nuclear extracts revealed high levels of NF-κB subunits p65 and p50 in wild-type IGFBP2 cells compared with low levels in parental and RGE-mutant cells (**Figure 19A**). NF-κB /DNA binding was additionally assessed via EMSA. Parental and RGE-mutant IGFBP2 cells exhibited low levels of DNA binding, whereas strong binding was observed in wild-type IGFBP2 extracts. Supershift assays confirmed that the binding was specific for p65 and p50 (**Figure 19B**). Luciferase assays demonstrated that NF-κB transcriptional activity was significantly increased in cells expressing IGFBP2 versus parental or RGE-mutant cells ($p<0.0001$; **Figure 19C**), validating that IGFBP2 activates NF-κB through integrin signaling.

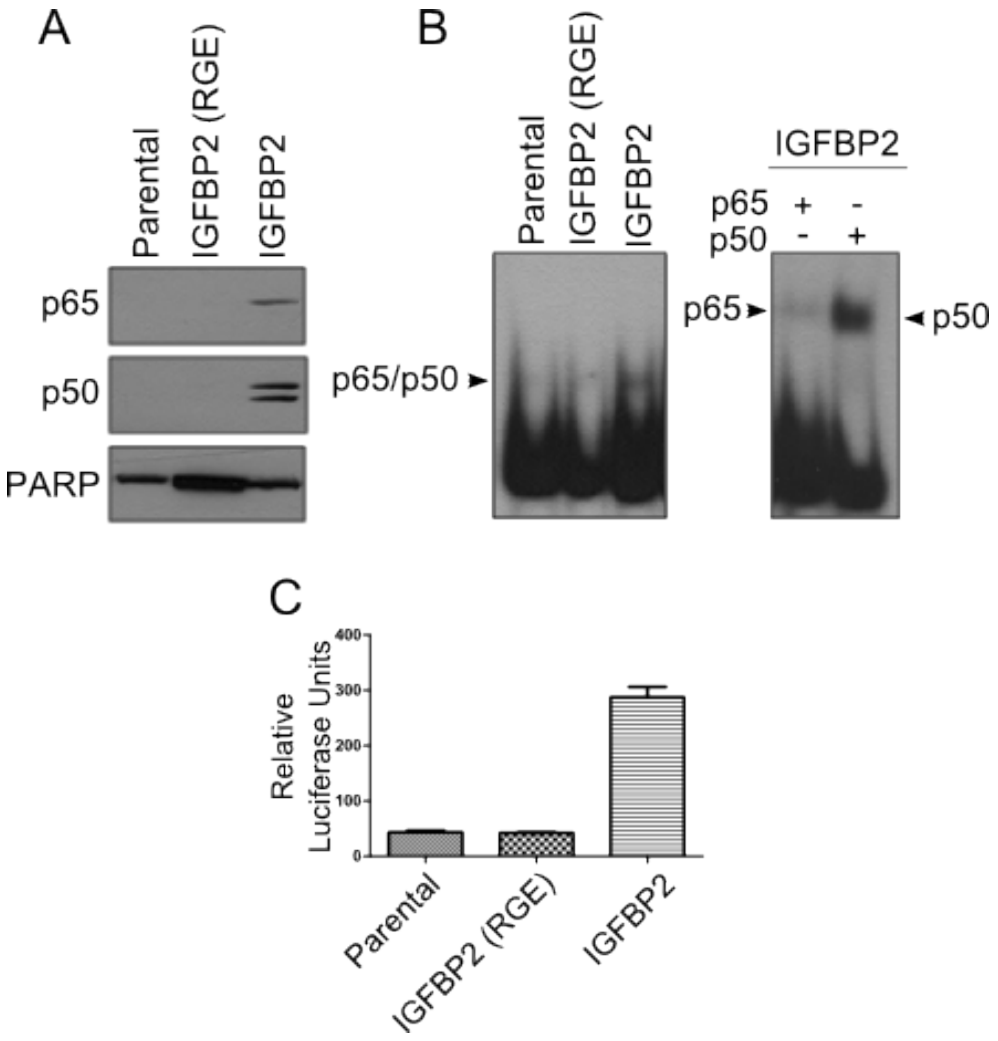


Figure 19. IGFBP2 induces NF-κB activity. (A) Western blot analysis of p65 and p50 from nuclear extracts from the indicated cells. (B) Nuclear extracts from (A) were subjected to EMSA using an NF-κB consensus probe. Supershift assays were performed to validate the presence of p65 or p50. (C) NF-κB activation, as assessed by luciferase assay, in the indicated cells. *p<0.0001, ANOVA with Tukey's multiple comparison test. Error bars represent SEM.

We next wanted to test cell viability after NF-κB inhibition in cells expressing high levels of IGFBP2 to determine if IGFBP2 rendered cells more sensitive to NF-κB inhibition. Primary mouse glial progenitor cells (GPCs) were obtained from Ntv-a transgenic mice at postnatal day 1 and treated with conditioned media from DF-1 cells engineered to overexpress RCAS particles containing PDGFB or IGFBP2. GPCs were then treated with PS-341, a proteasome inhibitor that affects NFκB by preventing the proteasomal degradation of its inhibitor, IκBα. Cell viability was measured via Trypan Blue staining, and cells were counted 24 hours after PS-341 treatment. Untreated cells and those infected with PDGFB alone were unaffected by NF-κB inhibition; in contrast, cells infected with both PDGFB and IGFBP2 had significantly reduced cell viability and sensitivity to PS-341, indicating that IGFBP2 promotes signaling pathways that activate NF-κB ($p<0.001$, ANOVA with Bonferroni's posttests) (**Figure 20A**). To confirm that the reduced viability after PS-341 treatment was specifically due to NF-κB inhibition, we used the same *ex vivo* approach but with a dominant-negative IκBα mutant (IκBαM) in lieu of PS-341. IκBαM contains S34,36A mutations, which prevent phosphorylation and subsequent IκBα degradation, inhibiting NF-κB activation. GPCs were infected with PDGFB alone or in combination with IGFBP2 or IGFBP2 and IκBαM. Measurement of cell viability via MTT assay confirmed that cells expressing IGFBP2 exhibited a significantly increased sensitivity to NF-κB inhibition ($p<0.01$, ANOVA with Bonferroni's posttests) (**Figure 20B**).

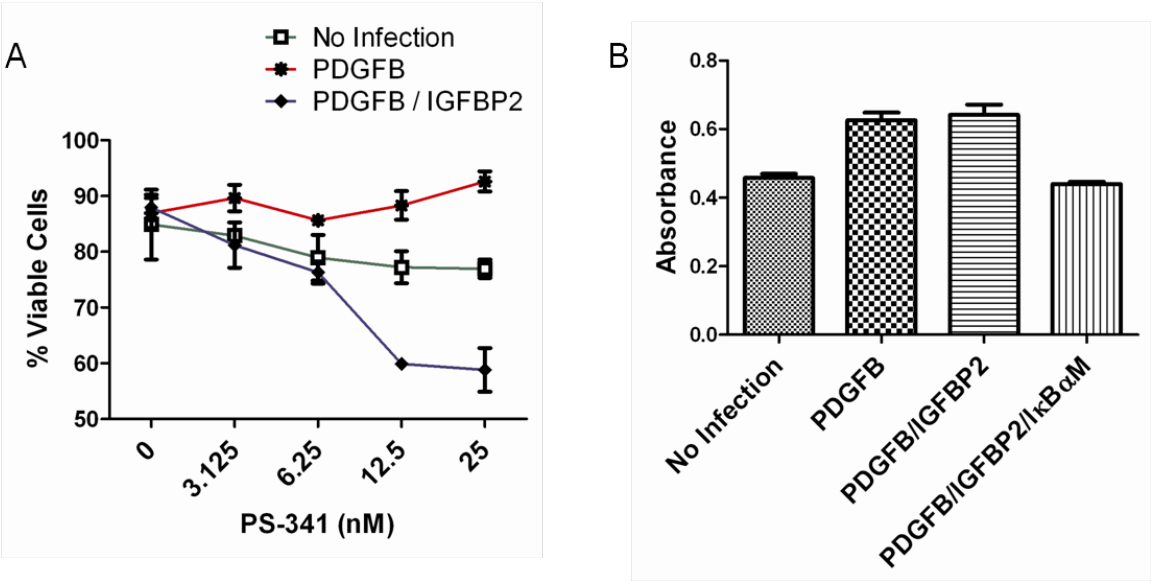


Figure 20. Elevated IGFBP2 expression sensitizes cells to NF-κB inhibition.
Primary glial progenitor cells from Ntv-a mice were infected with RCAS particles expressing the indicated gene. (A) Cells uninfected (control) or infected with RCAS-PDGFB alone or with IGFBP2 were treated with PS-341 for 24 hr, followed by Trypan Blue staining to assess cell viability. *p<0.001, ANOVA with Bonferroni's posttests. (B) MTT assay 24 hr after infection with RCAS-PDGFB, PDGFB with IGFBP2, or PDGFB with IGFBP2 and IκBαM. *p<0.01, ANOVA with Bonferroni's posttests. Error bars indicate SEM.

Integrin, ILK, and NF-κB regulate IGFBP2-induced glioma progression

Rationale. We have demonstrated that IGFBP2 activates integrin β1, requires ILK for Rac activation and cell motility, and activates NF-κB. We next wanted to test whether these pathways play a critical role in glioma progression under physiological conditions, in a glioma mouse model which is initially tumor-free. We utilized the RCAS/Ntv-a glial-specific transgenic mouse model to test our hypothesis *in vivo*. This system uses an avian retrovirus, RCAS, to infect cells expressing the avian tv-a receptor (80). Ntv-a mice have been engineered to express the tv-a receptor under the control of the nestin promoter (82). Nestin directs tv-a receptor expression to glial progenitor cells, leaving the remaining resident cells unaffected upon RCAS infection. RCAS constructs containing the gene of interest are transfected into avian DF-1 fibroblast cells and subsequently injected intracranially into Ntv-a mice on postnatal day 1. If the gene or combination of genes is oncogenic, mice form gliomas that closely resemble human glioma in their pathogenic features, including perinuclear satellitosis, perinuclear halos in oligodendroglioma, vascular proliferation, and pseudopalisading necrosis (86, 398). An experimental schematic is depicted in **Figure 21**.

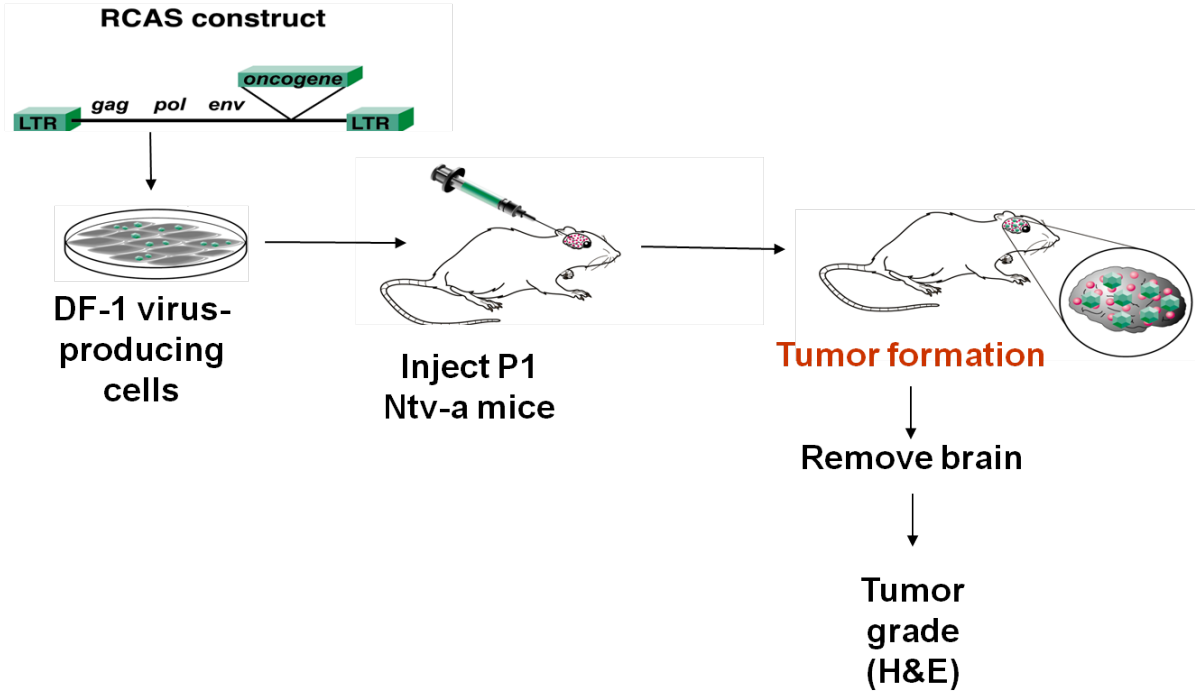


Figure 21. Experimental procedure for mouse model studies. RCAS constructs carrying the gene of interest are transfected into avian DF-1 cells which propagate the retrovirus. The cells are then injected into the right hemisphere of postnatal day 1 Ntv-a mice and subsequently monitored for tumor formation, upon which the brain is removed, fixed, and stained with H&E for pathological analysis.

Using this mouse model, we previously demonstrated that intracranial injection of PDGFB led to the development of low-grade diffuse glioma (LGDG) in the majority of mice. Co-delivery of IGFBP2 and PDGFB resulted in the development of high-grade diffuse glioma (HGDG) in nearly half of the mice (88). We therefore used this glioma progression model to investigate whether IGFBP2/integrin binding, ILK, and NF-κB are physiologically active pathways *in vivo*, and their role in IGFBP2-mediated glioma progression.

Results. To test whether IGFBP2/integrin binding was required for progression, we injected RCAS-PDGFB alone or in combination with RCAS vectors encoding either wild-type IGFBP2 or the RGE-mutant IGFBP2. PDGFB alone led to glioma formation in more than 83% of the mice; in 11% of these mice, the gliomas showed vascular proliferation and/or foci of necrosis, which qualified them as HGDG lesions. Co-delivery of wild-type IGFBP2 and PDGFB produced gliomas in 72% of the mice, of which 44% were classified as HGDG, similar to our previous study (88). Strikingly, co-delivery of RGE with PDGFB resulted in a 78% glioma incidence, but only 4% of these were classified as HGDG, indicating that the integrin-binding function of IGFBP2 is critical for its ability to drive glioma progression (**Figure 22A-D**).

We next tested whether ILK could produce an effect similar to that of IGFBP2 and whether kinase-dead ILK (ILK-KD) was capable of blocking IGFBP2-mediated progression. Wild-type ILK or ILK-KD (S343A) was injected with PDGFB. Wild-type ILK led to a significant increase in HGDG incidence compared to injection of PDGFB alone (from 11% to 44% , p=0.006), with the same proportion of anaplastic progression as the PDGFB and IGFBP2 combination. In contrast, ILK-KD combined with PDGFB did not produce a significant increase in HGDG incidence (14% vs. 11%, p=0.750). When ILK-KD was co-injected with IGFBP2 and PDGFB, an 18% HGDG incidence was observed, which also was not statistically different from PDGFB alone, indicating that, indeed, ILK is a critical downstream effector of the IGFBP2 pathway (**Figure 22A-D**).

We determined the role of NF-κB in IGFBP2-induced glioma progression by co-injecting a mutant form of IκBα (IκBαM; S32,36A) with PDGFB and IGFBP2 or PDGFB and

ILK. IκBαM inhibited NF-κB activation by retaining it in the cytoplasm. Strikingly, inhibiting NF-κB in the IGFBP2 combination produced LGDG tumors in all the mice, suggesting that IGFBP2 requires NF-κB to induce progression. Similarly, inhibiting NF-κB in the ILK combination also prevented progression (22% grade 3 incidence; p=0.42 vs. PDGFB alone).

Thus, IGFBP2 drives glioma progression through integrin signaling, ILK, and NF-κB; genetically blocking any step of the pathway robustly prevents glioma progression, highlighting the potential therapeutic value of these findings (**Figure 22D**).

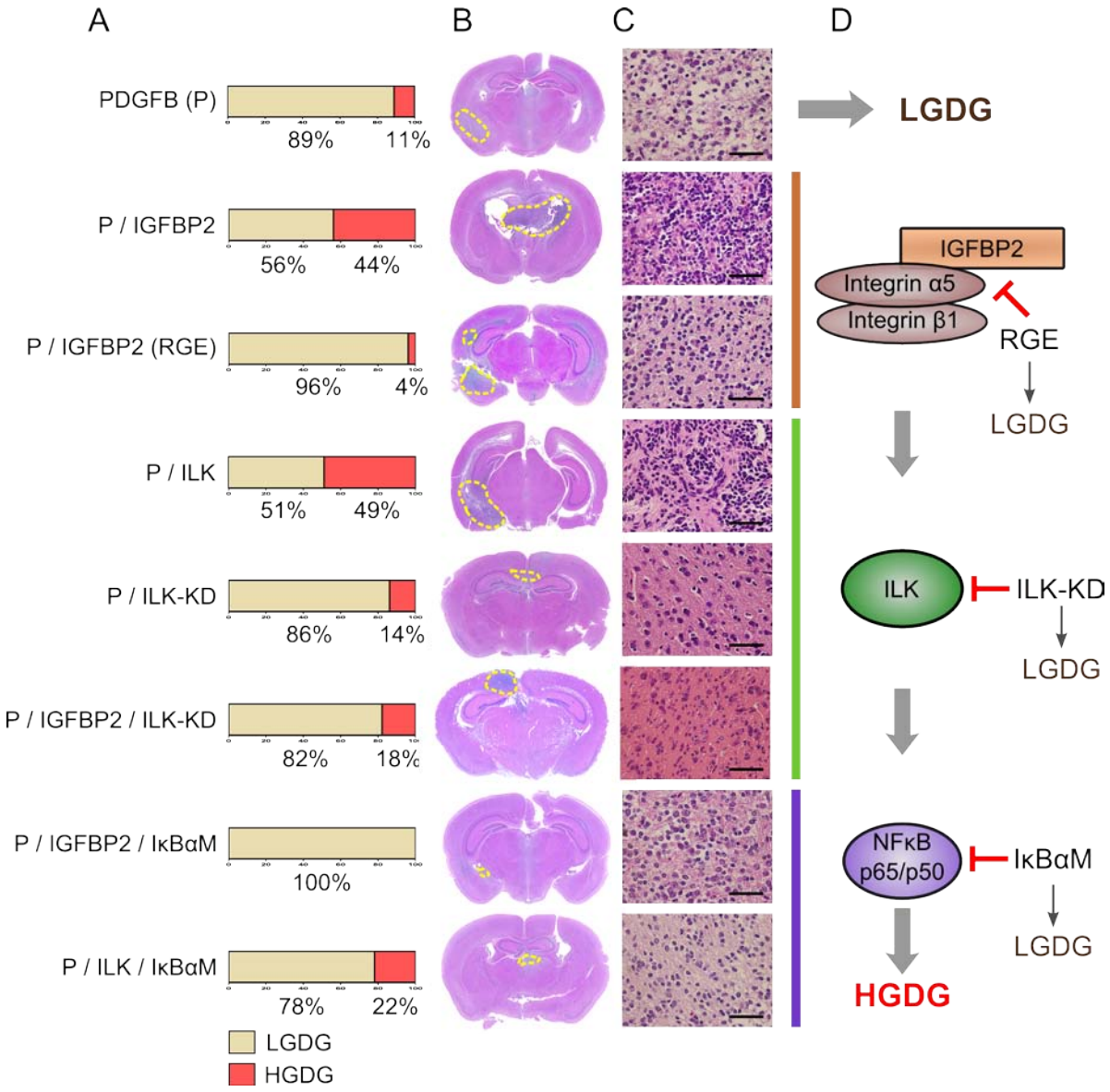


Figure 22. IGFBP2 drives glioma progression by activating the integrin/ILK/NF-κB network. (A) The percentage of mice from each RCAS injection combination with LGDG or HGDG tumors. (B) Representative whole-brain sections from each injection combination. The dotted line indicates the tumor. (C) Representative H&E sections from tumors generated from each RCAS injection combination. Scale bar, 50 μm. (D) Model of IGFBP2-induced glioma progression. IGFBP2 lies at the top of a signaling cascade that requires integrin binding, ILK, and p65/p50 to induce glioma progression (HGDG). Disruption of integrin binding to IGFBP2, inhibition of ILK kinase activity, or inhibition of p65/p50 blocks IGFBP2's ability to induce glioma progression (LGDG tumors are produced).

RCAS Injection Combination	Tumor Incidence	HGDG Incidence	*p-value
PDGFB	83% (35/42)	11%	-
PDGFB/IGFBP2	72% (36/50)	44%	0.003
PDGFB/IGFBP2 (RGE)	78% (25/32)	4%	0.389
PDGFB/ILK	79% (37/47)	49%	<0.001
PDGFB/ILK-KD	81% (21/26)	14%	1.000
PDGFB/ILK-KD/IGFBP2	77% (17/22)	18%	0.669
PDGFB/IGFBP2/IκBαM	74% (14/19)	0%	0.311
PDGFB/ILK/IκBαM	90% (18/20)	22%	0.420

*Fisher's Exact Test, compared to PDGFB only group

Table 3. Tumor distribution of Ntv-a mice.

CHAPTER 4. Discussion

Summary

Gliomas represent one of the most deadly types of cancer. Patients with the most commonly diagnosed form of glioma, GBM, typically survive a median of 15 months with the standard of care treatment (8). The identification and elucidation of complex oncogenic signaling networks is necessary to discover potential therapeutic targets that can be translated into clinical treatments. Although IGFBP2 is acknowledged as an important oncogene promoting glioma progression, the signaling pathway and mechanism of action remained under-characterized. In this current study, we combined transcriptional profiling of human glioma, biochemical analysis and perturbation of IGFBP2-associated pathways, and a spontaneous glioma mouse model to assess the clinical significance of IGFBP2 in relation to the integrin and ILK pathways and to NF-κB transcriptional regulation of invasion-related target genes. Our results provide evidence that IGFBP2 lies at the top of a signaling cascade involving integrins α5/β1, ILK, and NF-κB. Further, blocking any constituent of this network prevented IGFBP2-driven glioma progression *in vivo* (89). Therefore, we have identified novel effectors of IGFBP2 that could open up new avenues for intervention in tumors with high IGFBP2 expression, not only for glioma, but also in a broad range of other cancer types.

The role of the IGFBP2 pathway in cell migration and invasion

Most cancer-related deaths result from the spread of cancer from the initial site to distant organs, a phenomenon known as metastasis. The ability of cells to metastasize greatly relies upon migration and invasion because cells must be able to both move and

invade the surrounding tissue by degrading the ECM. Although primary brain tumors rarely metastasize, their ability to invade the surrounding parenchyma is a key feature which contributes to the morbidity of the disease. Both oligodendroglioma and astrocytoma (including GBM) are referred to as diffuse glioma, and are characterized by individual diffusely infiltrative cells. This feature produces an ill-defined tumor border that precludes complete surgical resection. As the extent of surgical resection is an important prognostic indicator (63), identifying major mechanisms by which cells migrate and invade away from the tumor mass is critical to provide more effective treatment**.** Many proteins are involved in these processes, including integrins, ILK, small GTPase proteins such as Rac, MMPs and others (399). Given that IGFBP2 has been associated with many of the major migration and invasion-promoting molecules, it is not surprising that the main function of IGFBP2 in glioma involves promoting cellular migration and invasion (156, 161). Previously, our group performed cDNA microarrays in IGFBP2-overexpressing glioma cells and found that migration and invasion-related genes were among the most striking differentially regulated alterations. In particular, the transcriptional activity of MMP-2 was increased in response to IGFBP2 (156). The relationship between IGFBP2 and MMP-2 has also been confirmed in human bladder cancer cells, which resulted in enhanced metastatic potential (158). In our previous study, we found that tumors that arose from *INK4a/ARF* loss and PDGFB overexpression had elevated endogenous IGFBP2 levels that were localized at the invasive front (94). Similarly, stereotactic biopsies of GBMs that progressed following bevacizumab treatment displayed a prominent infiltrative growth pattern, accompanied with strong IGFBP2 and MMP-2 staining (162), providing compelling evidence that IGFBP2 plays a dominant role in tumor invasion *in vivo*. Current therapies are cytotoxic and do not affect the migratory potential of cells. Given that IGFBP2 is a strong promoter of migration and

invasion processes, therapeutic targeting of IGFBP2 in combination with standard treatments could both eliminate tumor cells and inhibit invasion into surrounding areas.

Functional significance of the IGFBP2/integrin relationship

IGFBP2 contains an integrin-binding RGD motif and has been confirmed to bind integrin α5b1. In the current study we confirmed that this interaction induced integrin β1 activation and affected a variety of pathways, most prominently migration and invasion. Integrins bind an array of proteins on either the extracellular domain or cytoplasmic tail. Integrin activation may occur by either an outside-in or an inside-out mechanism; however, it remains unknown which type of integrin signaling IGFBP2 participates in. IGFBP2 is both a secreted and intracellular protein, and there appears to be an active trafficking system for IGFBP2 going in and out of cells. We have observed that addition of purified IGFBP2 protein to the media of low-IGFBP2 expressing cell culture results in import of IGFBP2 into the cells. Thus, it is conceivable that IGFBP2 may bind to both extracellular and cytoplasmic portions of the integrins. The extracellular portion of integrin binds to matrix proteins and is important for cell adhesion. Increased IGFBP2 in the media actually increased cell adhesion, suggesting that IGFBP2 does not compete for binding of matrix, if in fact IGFBP2 does bind to the extracellular portion of the integrin. The integrin cytoplasmic tail binds essential proteins for activation, such as talin and ILK (206). It is plausible that IGFBP2 binds the integrin α5 cytoplasmic tail, leading to recruitment of talin and ILK to initiate inside-out mediated cellular migration. In previous studies, overexpression of IGFBP2 led to upregulation of integrins, including α5, α5, and β1 (134, 156). In our current study, microarray analysis comparing RGE-mutant and wild-type IGFBP2 revealed that disruption of IGFBP2/integrin binding led to down-regulation of multiple integrins, indicating

that IGFBP2 strongly modulates integrin signaling. Additionally, a possible positive feedback loop occurs in which IGFBP2 binds and activates integrins, and in turn, induces their transcription.

Although IGFBP2 had been shown to promote cell migration through an integrin $\alpha 5$ interaction, the full functional significance of this interaction has been unclear. In our study, disruption of IGFBP2/integrin binding in glioma cells lines affected multiple pathways, most notably the cell motility and invasion pathways. The functional significance of the IGFBP2/integrin interaction was not only present *in vitro*, but was also essential *in vivo*, as evidenced by the fact that RGE-mutant IGFBP2 could no longer cooperate with PDGFB to induce glioma progression. These data closely reflected human glioma data, in which IGFBP2 expression is intimately linked with genes in the integrin pathway. Importantly, activation of this pathway is also significantly correlated with prognosis.

IGF-independent function of IGFBP2

It is now well-recognized that IGFBP2 has both IGF-dependent and IGF-independent functions (111, 122). IGFBP2 is involved in many important cell processes, including cell proliferation; however the proliferative effects are varied and may be mediated via IGF-dependent or IGF-independent mechanisms. IGFBP2 has been reported to increase proliferation of a number of cancer cell lines, including breast cancer (147) and prostate cancer cells (400). In the SNB19 cell lines used for this study, we have not found IGFBP2 to increase cell proliferation *in vitro* (156). In fact, a negative effect of IGFBP2 on proliferation has been reported in other studies (124, 126, 127). Our previous study of IGFBP2 in the RCAS/Ntv-a model revealed that gliomas arising from IGFBP2 (with PDGFB) exhibited increased tumor cell proliferation via phospho-Histone H3 staining (88). Overall,

our *in vivo* system shows a positive proliferative effect from IGFBP2, but our cell culture system does not. It appears that IGFBP2 cell proliferation effect is complex and is dependent on extracellular environmental conditions.

The possibility exists that IGFBP2 binding to integrin negatively impacts IGF-dependent functions. However, IGFBP2-integrin binding in MCF-7 breast cancer cells did not affect IGFBP2 binding to IGFs (165). Consistently, a study in neuroblastoma SHEP cells reported that mutation of the IGFBP2 RGD (to RGE) had no effect on the affinity of IGFBP2 for IGFs (125). Thus, it is unlikely that the RGE-mutant IGFBP2 decreased cell migration effects were due to a switch to increased IGF binding. In our work, pathway analysis from the microarray experiment did not reveal significant alteration in the IGF pathway between cells expressing the RGE mutant and wild-type IGFBP2. Based on these observations, we do not believe that an IGF-dependent function is a key player in our experimental systems; on the other hand, a recent publication reported that IGFBP2 recruits endothelia cells via activation of IGF-I/IGF-IR (169). More detailed and extensive experiments are required to assess the role of the IGF system in IGFBP2-mediated glioma progression.

Integrin-linked kinase as a key mediator in glioma progression

Strong ILK expression has been reported in many human malignancies and is inversely correlated with survival in melanoma and non-small cell lung, pancreatic, ovarian, and prostate cancers, but it had not previously been reported in glioma (260). In this study, we demonstrated that IGFBP2 requires ILK to induce Rac activation and subsequent cell migration. Our study confirms ILK to be an important factor in glioma progression. In our glioma mouse model, we demonstrated that ILK could replace IGFBP2 to drive glioma

progression, and that the kinase activity of ILK is required for IGFBP2-mediated glioma progression. Thus, it appears that IGFBP2 binds integrin, inducing integrin clustering and ILK recruitment, which activates Rac and mediates cell morphological changes that facilitate cell movement. Targeting ILK activity in glioma with increased IGFBP2 expression may have a clinical benefit, since these functional roles appear to be clinically relevant. We found that IGFBP2 expression in human glioma samples significantly associates with expression of ILK pathway genes, which is also reflected by the decreased survival duration among patients exhibiting elevated expression of both IGFBP2 and ILK.

Consistent with the observation that IGFBP2 and ILK expression levels were highly correlated in human glioma samples, we observed an increase in ILK protein levels in cells stably expressing IGFBP2 compared to parental cells. Similarly, a separate study also reported that overexpression of IGFBP2 in a breast cancer cell line led to increased ILK expression (390). Here we have demonstrated that IGFBP2 activates integrin β1, which is consistent with activation of ILK. However, why ILK expression is increased is not yet clear. Since putative NF-κB binding sites are present in the ILK promoter, it is conceivable that there is a positive regulatory loop whereby IGFBP2 induces NF-κB activation, which then binds the ILK promoter to increase ILK expression.

The role of NF-κB and potential regulatory loop with IGFBP2

NF-κB is constitutively active in glioma and has been implicated as in important oncogene through a variety of mechanisms. The genetic loss of *NFKBIA* is one major mechanism by which NF-κB is activated (372). In addition, NF-κB appears to cooperate with EGFR and Akt in driving glioma progression (156, 372). We demonstrated that IGFBP2 indeed activates NF-κB, and IGFBP2-expressing cells were more sensitive to NF-κB

inhibition. Disruption of IGFBP2/integrin binding strongly attenuated NF-κB activation, indicating that IGFBP2 activates NF-κB through integrin engagement. Blocking NF-κB robustly prevented IGFBP2- and ILK-mediated glioma progression, underscoring the potential therapeutic benefit of NF-κB inhibition in tumors that highly express IGFBP2. Since NF-κB exerts its function at the bottom convergence point of the integrin and ILK pathway, inhibiting NF-κB may present the most rationale target in the IGFBP2 signaling cascade. However, additional investigation will be required to determine the precise mechanism by which NF-κB is activated in this pathway. Since Akt is a direct target of ILK and is a known NF-κB activator via IKK phosphorylation, Akt is a logical molecule responsible for inducing NF-κB activation in the IGFBP2 pathway. However, caution must be used when administering anti-NF-κB therapies, due to the potential hazards of immunosuppression with long-term use.

Not only does IGFBP2 activate NF-κB, but evidence exists that NF-κB also regulates IGFBP2. Putative NF-κB binding sites have been identified on the IGFBP2 promoter in rat alveolar cells, and hyperoxia led to NF-κB activation and subsequent induction of IGFBP2 transcriptional activity (401). We have also observed that activated NF-κB results in increased IGFBP2 protein levels in glioma cells. These findings could indicate the presence of a positive feedback loop that perpetuates signaling events contributing to progression, in which IGFBP2 would activate NF-κB, which would increase IGFBP2 transcription. It has also been reported that mitogens induce an INK4a/NF-κB-p65 interaction, which results in decreased transactivation activity of NF-κB (402). Further, *INK4a* mutations have been reported to attenuate the inhibitory function on the NF-κB promoter (403). In Ntv-a *INK4a/ARF*$^{-/-}$ mice with PDGFB-driven glioma, increased IGFBP2

protein expression was observed after loss of *INK4a/ARF*, and downregulation of endogenous IGFBP2 led to a survival benefit. Therefore, it is plausible to hypothesize that loss of *INK4a/ARF* would increase NF-κB activity, leading to elevated transcription of IGFBP2 and, thereby, potentiate glioma progression.

IGFBP2 represents a physiologically active signaling pathway

In this study, we demonstrated that IGFBP2 activates integrin β1 and its downstream pathways, requires ILK to induce cell migration, and activates an NF-κB invasion-related transcriptional program. The most compelling evidence was obtained from *in vivo* studies that illustrate that these relationships represent true physiologically active signaling pathways. Although cell culture experiments and xenografts experiments answer key, direct questions, better systems are required to ultimately test the clinical applicability and significance of potential therapeutic targets. The RCAS/Ntv-a spontaneous glioma mouse model has enabled us to more closely recapitulate human glioma in a physiologically relevant setting by transforming normal murine glial cells with combinations of clinically relevant oncogenes (404). This study is the first to dissect an entire signaling pathway in the RCAS/tv-a system. We validated that IGFBP2-mediated glioma progression is driven by an integrin/ILK/NF-κB network and most importantly, we demonstrated that genetic inhibition of each pathway component thwarted glioma progression.

Therapeutic implications

This study provides strong evidence that targeted inhibition of the IGFBP2 pathway may produce a clinical benefit for patients. The current standard therapy involves the alkylating agent temozolomide with concurrent radiotherapy (57). Although a study has

reported that the IGFBP2 antisense drug OGX-225 attenuated tumor growth in a breast cancer xenograft model (147), a follow-up investigation has not been performed, and no other IGFBP2 inhibitors are available. However, integrin (405), ILK (198, 325), and NF-κB (406) are druggable targets that have been used in preclinical studies and clinical trials. Particularly, the cyclic RGD-mimetic αv integrin inhibitor cilengitide is in phase III clinical trials in glioma, testing its efficacy in combination with temozolomide and radiotherapy in newly diagnosed GBM with hypermethylated *MGMT* promoter (67). Although the results have been encouraging, perhaps stratifying patients with the IGFBP2 gene signature would elicit a more favorable clinical response. Three β1 inhibitors are currently in clinical trials for head and neck tumors and advanced solid tumors, including volociximab, which is a humanized monoclonal antibody against integrin α5β1 (407). The β1 integrin inhibitors may be effective in glioma, since we specifically demonstrated its requirement for IGFBP2-oncogenic function. Small molecule pharmacological inhibition of ILK in glioma xenografts has produced favorable outcomes. In U87MG xenografts, ILK inhibition was shown to result in significant delayed tumor growth, in addition to decreased angiogenesis and vasculature functionality (296, 317). The combined treatment of the ILK inhibitor QLT2054 and gemcitabine produced synergistic effects in primary pancreatic cancer xenografts (323), and may also perform similarly with temozolomide in GBM patients. To date, no ILK inhibitors have been tested in the clinic for any type of cancer. The proteasomal NF-κB inhibitor bortezomib has thus far only demonstrated efficacy in multiple myeloma patients (382), although it has not been tested on glioma in the clinic. NF-κB inhibitors have promoted glioma stem cell senescence and sensitized glioma cells to chemotherapies, indicating potential therapeutic benefit (375, 376, 379). Drugs targeting the IGFBP2 network could be used either in combination or with current standard therapy.

In summary, IGFBP2 is a strong, clinically relevant oncogene that exploits an integrin/ILK/NF-κB network to create the major driving force behind glioma progression. We are hopeful that by inhibiting the IGFBP2 signaling pathway, this will result in tumor regression and a subsequent survival benefit, not only for glioma patients but also for patients with a broad range of other cancers.

CHAPTER 5. Future Directions

Translational application of IGFBP2 network inhibition

This thesis has provided a basis for various avenues of future research. In particular, the RCAS/tv-a mouse model used in this work is an optimal preclinical system to test the efficacy of integrin, ILK, and NF-κB inhibitors in tumors with high IGFBP2 expression. Since previous investigations in our lab revealed that IGFBP2 was responsible, in part, for shortened survival in *INK4A/ARF*$^{-/-}$ mice with PDGFB-driven tumors, current studies are ongoing to assess whether IGFBP2 gene ablation will prolong survival or block tumor progression. This will provide further evidence of the potential therapeutic benefit of IGFBP2 inhibition. As described in the **Discussion**, various β1 integrin inhibitors are available, and may be more effective in tumors with high IGFBP2 expression than the current integrin inhibitor cilengitide that is currently in clinical trial, and targets integrin αv. We are planning collaborations with Shoukat Dedhar's laboratory to test the ATP-mimetic ILK small molecule inhibitor, QLT0267, in PDGFB-injected mice with IGFBP2 or ILK. Given the effectiveness of this inhibitor in xenograft models in glioma and other cancers, anti-tumor results in our model would provide further framework to test ILK inhibitors in the clinic.

Determining the role of the IGF system in IGFBP2 oncogenic functions

Although our current results do not seem to indicate a major IGF-dependency on IGFBP2 functions, we cannot rule out the possibility that there is some IGF-dependency. We plan to test whether IGFBP2-dependent cell proliferation and cell migration are affected by the IGF system. This can be achieved by evaluating the cellular response to varying

molar ratios of IGF-I or IGF-II with IGFBP2. Additionally, the effects of the IGF system can be eliminated via knockdown of IGF-IR and IGF-IIR. Finally, we plan to test the oncogenic capacity of IGF-I and IGF-II in our *ex vivo* system and mouse model. When injected alone, IGFs may not be sufficient to induce transformation. However, if IGFBP2 binding potentiates IGF function, as has been observed in some cases, LGDG should develop. In contrast, co-injection of IGF-I/II with PDGFB and IGFBP2 should prevent HGDG, if in fact IGF/IGFBP2 binding is inhibitory.

Identification of NF-κB activation mechanism

We have documented that IGFBP2 activates NF-κB via integrin activation, although we have not yet tested the precise mechanism. Since ILK is known to activate Akt, which in turn can phosphorylate and activate the IKK complex, it is plausible that Akt is required to activate NF-κB. In fact, Makino *et al*. (397) reported that ILK upregulated IKKα/β and activated NF-κB through ILK-dependent Akt phosphorylation. Additionally, we and others have found that IGFBP2 activates the Akt pathway (88, 166). We have tested the oncogenic capacity of the 3 Akt isoforms in the RCAS/Ntv-a mice, and found that Akt2 and Akt3 potently drive glioma progression; therefore, Akt2 and Akt3 are the likely Akt isoforms involved in NF-κB activation. To determine whether IGFBP2 requires Akt to induce NF-κB activation, Akt2 and Akt3 can be injected either with IκBαM or with dominant negative IKK. If Akt requires NF-κB activation to promote glioma progression, the addition or IκBαM or dominant negative IKK will prevent development of HGDG. Next, a dominant negative Akt should block IGFBP2-induced progression if Akt is a key player in the IGFBP2 pathway to activate NF-κB.

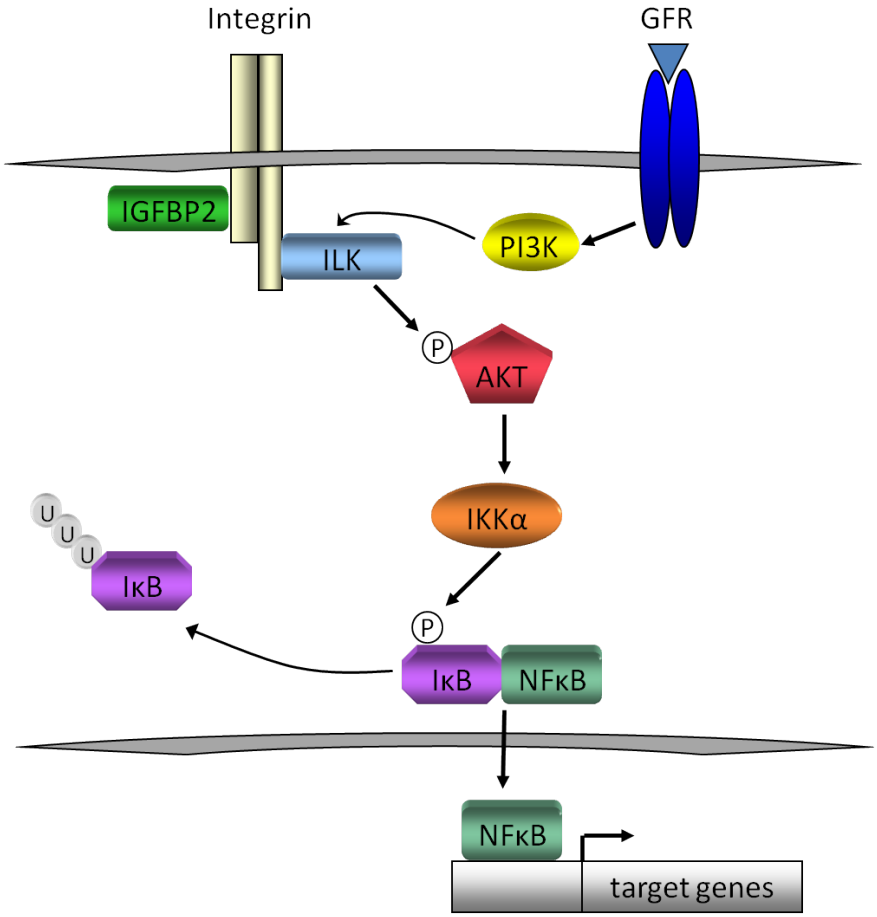
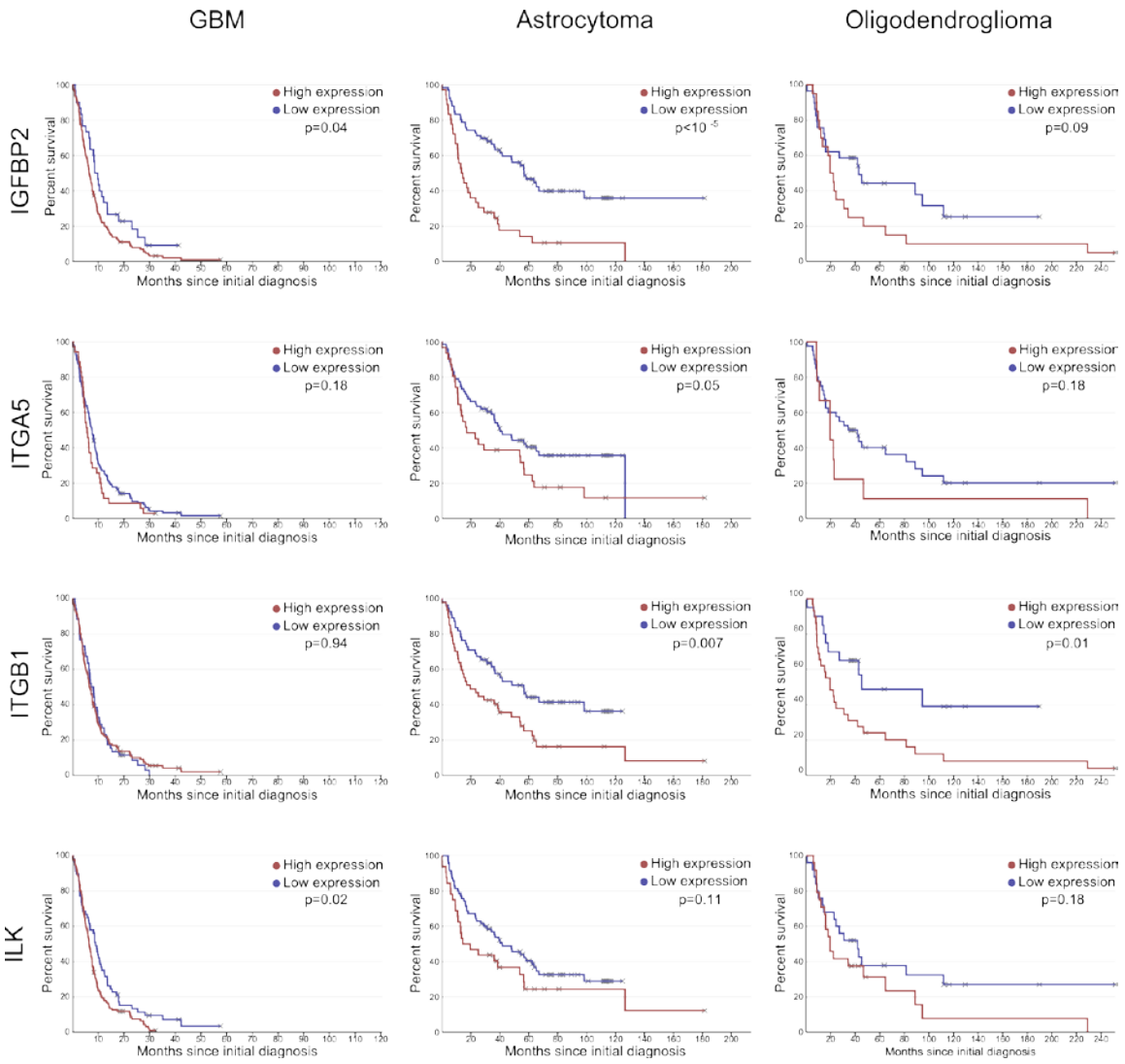


Figure 23. Proposed mechanism of IGFBP2-mediated NF-κB activation. Future studies will examine the involvement of Akt and the IKK complex in the IGFBP2 signaling pathway.

Appendix



Appendix Figure A1. IGFBP2, ITGA5, ITGB1, and ILK Kaplan-Meier survival plots according to glioma histology. Data was obtained from the Rembrandt database. P-values are derived from the log-rank test.

Appendix Table A1. Signaling pathways associated with IGFBP2 expression

Pathways	-log(p-value)	Ratio	Molecules
Agrin Interactions at Neuromuscular Junction	7.38E00	3.62E-01	GABPB1, ACTA2, LAMC1, JUN, ERBB4, LAMB1, ERBB2, ACTG2, DAG1, ITGA4, ITGB1, PXN, NRAS, RRAS, ACTB, ITGA2, LAMA2, ITGA5, ITGA3, ACTG1, ITGB3, PAK3, NRG3, MAPK10, PAK7
Integrin Signaling	7.05E00	2.34E-01	RAP2B, DIRAS3, ARPC5, PARVB, ARF4, ARPC4, CAV1, ARPC1A, ACTG2, PPP1CA, TSPAN4, ITGA4, CAPN5, AKT2, RRAS, ITGA5, RHOJ, MYL12A, RND3, MYL12B, ARPC2, ZYX, PAK7, ACTN4, ITGA7, RAP1B, RAP2A, RALA, TSPAN7, ACTA2, ILK, SHC1, ARF6, AKT1, ACTR3, VCL, VASP, ACTN1, ITGB1, PXN, NRAS, RHOC, ACTB, ITGA2, ITGA3, ACTG1, ITGB3, CAPNS1, PAK3
Cell Cycle: G2/M DNA Damage Checkpoint Regulation	6.29E00	3.67E-01	TP53, CDC25C, CKS2, CDK7, WEE1, CCNB2, PLK1, RPRM, CDK1, SKP2, CHEK1, CCNB1, GADD45A, CKS1B, TOP2A, BTRC, BRCA1, CHEK2
Caveolar-mediated Endocytosis Signaling	5.38E00	2.71E-01	ITGB1, ACTB, ITGA2, ACTA2, ITGA5, RAB5B, ITGA3, COPB1, ACTG1, ITGB3, COPB2, DYRK3, FLOT2, HLA-A, FLNA, FLNC, CAV1, HLA-B, ACTG2, PTRF, ITGA7, HLA-C, ITGA4
Axonal Guidance Signaling	4.93E00	1.69E-01	PFN1, MYL6, ARPC5, SEMA6B, TUBB, VEGFA, ARPC4, ABLIM3, PLCB1, TUBA1C, ARPC1A, FZD2, RTN4R, GNG12, ITGA4, AKT2, RRAS, ITGA5, MYL9, MYL12A, SRGAP3, SDCBP, MYL12B, ARPC2, FZD6, PAK7, FZD5, PDGFD, EPHA2, GNAL, NRP1, RAP1B, LRRC4C, RND1, PLXNA3, SLIT1, UNC5A, PDGFA, FZD1, ABLIM1, EFNB2, SHC1, ACTR3, AKT1, PPP3CB, SMO, ADAM19, PRKCE, SEMA4A, ERBB2, SHANK2, SEMA3F, VASP, PPP3CA, BMP1, ITGB1, SEMA3G, PXN, NRAS, CXCR4, ITGA2, PFN2, GNAI1, ITGA3, PLXND1, GNG5, GNAI3, PAK3, LINGO1, PRKAG2, SEMA4G, ADAM9, FZD7
Actin Cytoskeleton Signaling	4.92E00	1.93E-01	FN1, PFN1, MYL6, PDGFA, ARPC5, ACTA2, IQGAP1, FGF13, SHC1, IQGAP2, ACTR3, CYFIP2, ARPC4, FGF12, ARPC1A, ACTG2, VCL, PPP1CA, ACTN1, GNG12, IQGAP3, ITGA4, ITGB1, PXN, NRAS, TMSB10/TMSB4X, RRAS, ACTB, ITGA2, PFN2, ITGA5, WASF1, ITGA3, ACTG1, MYL9, MYL12A, PAK3, MYL12B, ARPC2, VAV3, CD14, MYH9, PAK7, ACTN4, PDGFD, MSN
Cell Cycle Control of Chromosomal Replication	4.82E00	3.87E-01	MCM5, MCM3, CDC45, RPA3, CDK7, CDC6, CDK6, CHEK2, MCM4, DBF4, CDK2, MCM7
Molecular Mechanisms of Cancer	4.59E00	1.7E-01	RAP2B, DIRAS3, NCSTN, CDKN2C, E2F6, PLCB1, BRCA1, HIPK2, NFKBIB, FZD2, TP53, AKT2, CASP3, RRAS, CDK6, RHOJ, AURKA, TCF3, RND3, MAPK10, FZD6, MAP2K3, FZD5, LEF1, PAK7, CFLAR, CDK2, GNAL, CAMK2G, RAP1B, RAP2A, RALA, CTNNA1, PSENEN, FZD1, FAS, CHEK1, CASP6, SHC1, SYNGAP1, JUN, AKT1, TGFB2, SMO, PRKCE, CHEK2, BMP1, ADCY2, CDC25C, NRAS, HAT1, RHOC, GNAI1, SMAD7, BAX, BAK1, APH1A, FADD, GNAI3, PAK3, RASGRF1, PRKAG2, CASP7, FZD7
p53 Signaling	4.56E00	2.6E-01	JMY, C12orf5, FAS, BIRC5, CHEK1, CASP6, AKT1, JUN, GADD45A, PPP1R13B, BRCA1, HIPK2, CHEK2, TP53, AKT2, THBS1, HDAC1, TNFRSF10B, RPRM, BAX, TP53I3, PCNA, SNAI2, CDK2, SIRT1
Regulation of Actin-based Motility by Rho	3.9E00	2.42E-01	PFN1, MYL6, RHOC, ACTB, DIRAS3, ACTA2, ARPC5, PFN2, WASF1, RHOJ, MYL9, MYL12A, ACTR3, RND3, PAK3, MYL12B, ARPC2, ARPC4, ARPC1A, PAK7, ACTG2, PPP1CA
Role of CHK Proteins in Cell Cycle Checkpoint Control	3.8E00	3.43E-01	TP53, E2F6, PCNA, CDC25C, RFC4, HUS1, RFC2, BRCA1, CDK1, CHEK2, CDK2, CHEK1
TNFR1 Signaling	3.74E00	2.83E-01	CASP3, TNFAIP3, IKBKE, FADD, TANK, CASP6, TRADD, MADD, JUN, RIPK1, PAK3, CASP2, PAK7, NFKBIB, CASP7
Hereditary Breast Cancer Signaling	3.63E00	2.09E-01	BARD1, DDB2, CHEK1, POLR2L, AKT1, GADD45A, HDAC11, RFC2, POLR2H, BRCA1, CHEK2, TP53, CDC25C, AKT2, NRAS, HDAC4, RRAS, WEE1, HDAC1, CDK6, CDK1, CCNB1, HDAC5, HDAC3, RFC4, POLR2E, BRCA2
Virus Entry via Endocytic Pathways	3.44E00	2.2E-01	ITGB1, NRAS, RRAS, ACTB, ITGA2, ACTA2, ITGA5, ITGA3, ACTG1, AP2S1, ITGB3, HLA-A, FLNA, FLNC, CLTCL1, CAV1, HLA-B, TFRC, PRKCE, ACTG2, ITGA4, HLA-C

Cyclins and Cell Cycle Regulation	3.39E00	2.25E-01	TP53, HDAC4, CDK7, WEE1, HDAC1, CDK6, CCNB2, CDKN2C, PPP2R5A, CDK1, SKP2, HDAC5, CCNB1, E2F6, CCNA2, HDAC3, HDAC11, TGFB2, BTRC, CDK2
Ephrin Receptor Signaling	3.38E00	1.76E-01	RAP1B, PDGFA, ARPC5, VEGFA, SHC1, EFNB2, AKT1, ACTR3, GRIN2C, ARPC4, ARPC1A, GNG12, ITGA4, ITGB1, AKT2, PXN, NRAS, RRAS, CXCR4, ITGA2, GNAI1, ITGA5, STAT3, ITGA3, GNG5, GRIN3A, GNAI3, SDCBP, ABI1, PAK3, ARPC2, PAK7, PDGFD, EPHA2, GNAL
ILK Signaling	3.08E00	1.87E-01	FN1, MYL6, DIRAS3, ACTA2, ILK, RICTOR, VEGFA, PARVB, TGFB1I1, JUN, AKT1, FLNA, CHD1, ACTG2, ACTN1, ITGB1, PXN, AKT2, FBLIM1, CASP3, TMSB10/TMSB4X, RHOC, ACTB, RHOJ, ACTG1, PPP2R5A, ITGB3, MYL9, RND3, FLNC, SNAI2, MAPK10, MYH9, LEF1, ACTN4, MMP9
Mitotic Roles of Polo-Like Kinase	3E00	2.5E-01	KIF23, CDC25C, ESPL1, CDC20, PTTG1, WEE1, PRC1, CCNB2, PLK1, PPP2R5A, CDK1, CCNB1, HSP90B1, PLK4, KIF11, CHEK2
Mechanisms of Viral Exit from Host Cells	2.93E00	2.67E-01	SNF8, CHMP2A, SH3GL3, ACTB, ACTA2, SH3GLB1, PRKCE, LMNB2, ACTG2, SH3GL2, ACTG1, LMNB1
Rac Signaling	2.91E00	1.87E-01	ITGB1, NOX4, NRAS, RRAS, ITGA2, ARPC5, ITGA5, WASF1, IQGAP1, ITGA3, CDK5R1, IQGAP2, JUN, ACTR3, CYFIP2, PAK3, ARPC2, ARPC4, CD44, PAK7, ARPC1A, ITGA4, IQGAP3
FAK Signaling	2.82E00	1.96E-01	ITGB1, CAPN5, AKT2, PXN, NRAS, RRAS, ACTB, HMMR, ACTA2, ITGA2, ITGA5, ITGA3, ACTG1, AKT1, CAPNS1, PAK3, PAK7, VCL, ACTG2, ITGA4
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.7E00	1.9E-01	IGFBP4, FN1, MYL6, PDGFA, ACTA2, FAS, VEGFA, COL1A2, TIMP1, CYP2E1, TGFB2, ECE1, TNFRSF11B, IL8, SMAD7, IFNGR2, IGFBP5, MMP2, BAX, MYL9, COL1A1, LY96, IGFBP3, MYH9, CD14, IL1RAPL1, MMP9, COL3A1
Sphingosine-1-phosphate Signaling	2.68E00	1.93E-01	AKT2, ADCY2, CASP3, RHOC, PDIA3, PDGFA, DIRAS3, GNAI1, RHOJ, CASP4, GNAI3, CASP6, PLCE1, AKT1, RND3, CASP2, CASQ1, CASP1, S1PR1, PLCB1, PDGFD, SMPD3, CASP7
Huntington's Disease Signaling	2.57E00	1.64E-01	HSPA6, CASP4, HSPA5, CDK5R1, HSPA4, CASP6, SHC1, POLR2L, JUN, AKT1, HSPA1L, HDAC11, CASQ1, CASP1, PRKCE, PLCB1, POLR2H, DNAJB1, GNG12, TP53, CAPN5, AKT2, HDAC4, YKT6, CASP3, SH3GL3, HDAC1, DNM3, BAX, UBE2S, GNG5, HDAC5, GRM5, HDAC3, CAPNS1, POLR2E, CASP2, GOSR2, CASP7
Inhibition of Angiogenesis by TSP1	2.46E00	2.56E-01	VEGFA, TP53, AKT2, AKT1, JUN, SDC1, CASP3, THBS1, MAPK10, MMP9
Amyloid Processing	2.42E00	2.32E-01	CAPN5, AKT2, CSNK1A1, NCSTN, PSENEN, CDK5R1, APH1A, AKT1, CAPNS1, PRKAG2, PRKCE, MAPT, BACE2
Clathrin-mediated Endocytosis Signaling	2.41E00	1.74E-01	PDGFA, ARPC5, ACTA2, SH3GLB1, SH3GL2, RAB5B, FGF13, VEGFA, ARF6, ACTR3, PPP3CB, FGF12, ARPC4, ARPC1A, ACTG2, PPP3CA, SNAP91, ITGB1, ACTB, SH3GL3, ITGA5, DNM3, ACTG1, AP2S1, ITGB3, ARPC2, CLTCL1, TFRC, PDGFD, MYO1E
PAK Signaling	2.33E00	1.78E-01	ITGB1, PXN, NRAS, MYL6, CASP3, RRAS, PDGFA, ITGA2, ITGA5, ITGA3, MYL9, SHC1, MYL12A, PAK3, MYL12B, MAPK10, PAK7, PDGFD, ITGA4
Apoptosis Signaling	2.33E00	1.98E-01	TP53, CAPN5, NRAS, CASP3, RRAS, LMNA, IKBKE, BAX, FAS, BAK1, CDK1, CASP6, CAPNS1, CASP2, PRKCE, SPTAN1, NFKBIB, CASP7, MCL1
Death Receptor Signaling	2.27E00	2.15E-01	CASP3, TNFRSF10B, IKBKE, FAS, TANK, FADD, CASP6, TRADD, RIPK1, CASP2, CFLAR, NFKBIB, CASP7, HSPB1
Germ Cell-Sertoli Cell Junction Signaling	2.22E00	1.74E-01	DIRAS3, ACTA2, ILK, CTNNA1, IQGAP1, TUBB, AKT1, TGFB2, TUBA1C, ACTG2, ACTN1, ITGB1, PXN, NRAS, RHOC, RRAS, ACTB, ITGA2, RHOJ, ITGA3, ACTG1, CDH2, PAK3, RND3, MAPK10, ZYX, MAP2K3, PAK7, ACTN4

IL-8 Signaling	2.17E00	1.61E-01	ANGPT2, DIRAS3, CXCR1, EIF4EBP1, IRAK1, VEGFA, HMOX1, AKT1, PRKCE, NFKBIB, GNG12, IL8, NOX4, AKT2, NRAS, RHOC, RRAS, GNAI1, IKBKE, RHOJ, MMP2, BAX, CSTB, GNG5, GNAI3, ARAF, RND3, MYL12B, MAPK10, MMP9, IRAK4
Glioblastoma Multiforme Signaling	2.15E00	1.65E-01	TSC1, PDIA3, PDGFA, DIRAS3, FZD1, E2F6, SHC1, AKT1, PLCE1, SMO, PLCB1, FZD2, TP53, AKT2, NRAS, RHOC, RRAS, CDK6, RHOJ, TCF3, RND3, FZD6, LEF1, FZD5, PDGFD, CDK2, FZD7
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	2.11E00	1.86E-01	AKT2, PXN, FCGR2A, ACTB, ACTA2, ARPC5, ACTG1, HMOX1, PLA2G6, ARF6, ACTR3, AKT1, VAV3, ARPC2, ARPC4, PRKCE, ARPC1A, ACTG2, VASP
Neuregulin Signaling	2.03E00	1.76E-01	ITGB1, AKT2, NRAS, RRAS, ITGA2, ITGA5, ITGA3, CDK5R1, TMEFF2, SHC1, HSP90B1, AKT1, NRG3, ERBB4, PRKCE, ERRFI1, ERBB2, ITGA4
Glioma Invasiveness Signaling	1.99E00	2.17E-01	NRAS, RRAS, RHOC, HMMR, DIRAS3, RHOJ, MMP2, ITGB3, RND3, TIMP1, CD44, PLAU, MMP9
Type I Diabetes Mellitus Signaling	1.98E00	1.74E-01	SOCS3, CASP3, SOCS2, IFNGR2, IKBKE, IRF1, FAS, IRAK1, FADD, TRADD, RIPK1, HLA-A, HLA-E, MAPK10, HLA-B, FCER1G, MAP2K3, HLA-G, NFKBIB, HLA-C, TNFRSF11B
TWEAK Signaling	1.94E00	2.31E-01	FADD, CASP6, TRADD, RIPK1, CASP3, IKBKE, NFKBIB, CASP7, TNFRSF12A
Neuroprotective Role of THOP1 in Alzheimer's Disease	1.92E00	1.85E-01	HLA-A, HLA-E, HLA-B, PRKAG2, SERPINA3, ECE1, MAPT, HLA-G, MMP9, HLA-C
Antigen Presentation Pathway	1.92E00	2.33E-01	PSMB9, CALR, HLA-A, HLA-E, PDIA3, HLA-B, PSMB8, HLA-G, TAP1, HLA-C
Phospholipase C Signaling	1.92E00	1.46E-01	RAP1B, RALA, MYL6, DIRAS3, RPS6KA3, SHC1, HMOX1, PLCE1, PPP3CB, HDAC11, PLA2G5, PLCB1, PRKCE, PPP1CA, PPP3CA, GNG12, ITGA4, ITGB1, ADCY2, HDAC4, NRAS, RHOC, RRAS, FCGR2A, ITGA2, HDAC1, ITGA5, RHOJ, ITGA3, GNG5, HDAC5, MYL9, PLA2G6, MYL12A, HDAC3, RND3, MYL12B, FCER1G
ATM Signaling	1.92E00	2.22E-01	TP53, CDC25C, JUN, GADD45A, MAPK10, CCNB2, BRCA1, CDK1, CHEK2, CDK2, CHEK1, CCNB1
CXCR4 Signaling	1.88E00	1.6E-01	MYL6, DIRAS3, AKT1, JUN, PRKCE, PLCB1, GNG12, AKT2, PXN, ADCY2, NRAS, CXCR4, RHOC, RRAS, GNAI1, RHOJ, GNG5, MYL9, GNAI3, MYL12A, RND3, PAK3, MYL12B, MAPK10, PAK7, ELMO1, GNAL
PI3K/AKT Signaling	1.85E00	1.57E-01	TP53, ITGB1, TSC1, CDC37, AKT2, NRAS, RRAS, ITGA2, ILK, ITGA5, IKBKE, ITGA3, PPP2R5A, EIF4EBP1, SHC1, HSP90B1, GYS1, AKT1, HLA-B, NFKBIB, ITGA4, MCL1
Induction of Apoptosis by HIV1	1.81E00	1.97E-01	TP53, CASP3, CXCR4, IKBKE, BAX, BAK1, FAS, FADD, TRADD, RIPK1, MAPK10, NFKBIB, TNFRSF11B
NF-κB Activation by Viruses	1.8E00	1.83E-01	ITGB1, AKT2, NRAS, RRAS, ITGA2, ITGA5, IKBKE, ITGA3, TNFRSF14, ITGB3, AKT1, RIPK1, PRKCE, NFKBIB, ITGA4
CDK5 Signaling	1.76E00	1.81E-01	ITGB1, LAMA5, ADCY2, NRAS, RRAS, CABLES1, ITGA2, ITGA3, PPP2R5A, CACNA1A, CDK5R1, LAMC1, LAMB1, PRKAG2, MAPT, PPP1CA, GNAL
HMGB1 Signaling	1.75E00	1.8E-01	IL8, AKT2, NRAS, RHOC, RRAS, HAT1, DIRAS3, IFNGR2, RHOJ, AKT1, JUN, RND3, MAPK10, MAP2K3, SERPINE1, TNFRSF11B, MYST4, PLAT

IL-6 Signaling	1.75E00	1.8E-01	IL8, NRAS, RRAS, IKBKE, STAT3, CEBPB, COL1A1, SHC1, JUN, MAPK10, CD14, MAP2K3, IL1RAPL1, TNFAIP6, MAPKAPK2, NFKBIB, TNFRSF11B, HSPB1
Cdc42 Signaling	1.74E00	1.55E-01	RALA, MYL6, ARPC5, IQGAP1, IQGAP2, ACTR3, JUN, HLA-A, ARPC4, HLA-B, ARPC1A, HLA-G, ITGA4, IQGAP3, HLA-C, ITGB1, ITGA2, ITGA5, ITGA3, MYL9, MYL12A, PAK3, MYL12B, HLA-E, ARPC2, FCER1G, MAPK10
RhoA Signaling	1.69E00	1.79E-01	PFN1, MYL6, ACTB, ACTA2, ARPC5, PFN2, WASF1, ACTG1, MYL9, RHPN2, MYL12A, LPAR6, RAPGEF2, ACTR3, MYL12B, ARPC2, ARPC4, ARPC1A, ACTG2, MSN
Role of BRCA1 in DNA Damage Response	1.67E00	1.97E-01	TP53, E2F6, RFC4, GADD45A, BARD1, RFC2, RBBP8, BRCA2, PLK1, BRCA1, CHEK2, CHEK1
Cell Cycle: G1/S Checkpoint Regulation	1.67E00	1.97E-01	TP53, E2F6, HDAC3, HDAC4, HDAC11, HDAC1, CDK6, TGFB2, BTRC, CDK2, SKP2, HDAC5
Acute Phase Response Signaling	1.65E00	1.57E-01	SOCS3, SERPING1, FN1, SOCS2, SERPINA3, IRAK1, C1R, TRADD, SHC1, HMOX1, JUN, SOD2, AKT1, SERPINA1, OSMR, SERPINE1, NFKBIB, TNFRSF11B, AKT2, NRAS, RRAS, C1S, IKBKE, STAT3, CEBPB, TCF3, RIPK1, MAP2K3
Notch Signaling	1.62E00	2.09E-01	DLL1, NOTCH3, CNTN1, DTX4, NCSTN, PSENEN, HES1, JAG1, APH1A
Leukocyte Extravasation Signaling	1.62E00	1.56E-01	RAP1B, MYL6, MMP14, ACTA2, CTNNA1, RAPGEF4, TIMP1, CYBA, PRKCE, ACTG2, VCL, ACTN1, VASP, ITGA4, ITGB1, PXN, CXCR4, ACTB, GNAI1, MMP2, ACTG1, F11R, GNAI3, ICAM3, VAV3, MAPK10, CD44, PECAM1, ACTN4, MMP9, MSN
Amyotrophic Lateral Sclerosis Signaling	1.54E00	1.51E-01	TP53, CAPN5, CASP3, GRIA2, CACNA1C, RAB5B, BAX, CACNA1A, GRIN3A, VEGFA, CACNA1E, CAPNS1, GRID1, GRIN2C, CASP1, GRIK2, PPP3CA, CASP7
Role of PKR in Interferon Induction and Antiviral Response	1.49E00	1.96E-01	FADD, TP53, AKT1, CASP3, IKBKE, MAP2K3, TRAF5, NFKBIB, IRF1
Role of NFAT in Cardiac Hypertrophy	1.48E00	1.4E-01	PDIA3, CSNK1A1, SHC1, AKT1, PLCE1, PPP3CB, HDAC11, TGFB2, PLCB1, PRKCE, PPP3CA, GNG12, ADCY2, AKT2, NRAS, HDAC4, RRAS, HDAC1, SLC8A3, GNAI1, GNG5, HDAC5, RCAN1, GNAI3, HDAC3, MAPK10, PRKAG2, MAP2K3, CAMK2G
Factors Promoting Cardiogenesis in Vertebrates	1.46E00	1.68E-01	NOX4, CDC6, FZD1, TCF3, NOG, FZD6, TGFB2, SMO, PRKCE, LEF1, FZD5, ACVR1C, FZD2, CDK2, BMP1, FZD7
Calcium Signaling	1.44E00	1.35E-01	RAP1B, RAP2B, RAP2A, MYL6, ACTA2, PPP3CB, GRIN2C, HDAC11, CASQ1, RYR1, TPM4, PPP3CA, CALR, HDAC4, CHRNA9, HDAC1, SLC8A3, GRIA2, TPM2, HDAC5, ATP2B2, GRIN3A, MYL9, RCAN1, HDAC3, PRKAG2, MYH9, CAMK2G
Cellular Effects of Sildenafil (Viagra)	1.41E00	1.46E-01	KCNN3, PDE2A, ADCY2, KCNN2, MYL6, PDIA3, ACTB, ACTA2, CACNA1C, ACTG1, CACNA1A, CACNG2, MYL9, MYL12A, PLCE1, CACNA1E, MYL12B, PRKAG2, PLCB1, MYH9, ACTG2, PPP1CA
Macropinocytosis Signaling	1.39E00	1.71E-01	ITGB1, NRAS, RRAS, PDGFA, ITGA5, RAB34, ITGB3, ARF6, ABI1, PRKCE, CD14, ACTN4, PDGFD
Protein Ubiquitination Pathway	1.35E00	1.42E-01	PSMB3, PSMA3, CDC20, PSMA7, DNAJC12, HSPA6, USP54, DNAJC10, PSMB8, HSPA5, TAP1, HSPA4, HSP90B1, HLA-A, HSPA1L, HLA-B, DNAJB1, BRCA1, PSMA2, PSMC2, HLA-C, PSMB4, PSMB9, PSME2, DNAJC2, UBE2S, PSMD8, HSPA12A, SKP2, PSMB2, DNAJB11, PSMD12, PSMA5, PSMA4, BTRC, USP46, UBE2J2, HSPB1, UBE2I
Endoplasmic Reticulum Stress Pathway	1.34E00	2.78E-01	CASP3, XBP1, HSPA5, EIF2AK3, CASP7
Gα12/13 Signaling	1.33E00	1.56E-01	AKT2, PXN, NRAS, MYL6, RRAS, CDH6, CDH18, IKBKE, CDH11, MYL9, MYL12A, LPAR6, CDH2, JUN, AKT1, MYL12B, VAV3, CDH10, MAPK10, NFKBIB
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	1.32E00	1.33E-01	SOCS3, SFRP2, FN1, PDGFA, PDIA3, CSNK1A1, IL17RC, FZD1, IRAK1, VEGFA, TRADD, AKT1, C5AR1, JUN, PLCE1, PPP3CB, SMO, PRKCE, PLCB1, TRAF5, NFKBIB, MAPKAPK2, FZD2, PPP3CA, TNFRSF11B, IL8, AKT2, NRAS, RRAS, IKBKE, CEBPB, STAT3, TCF3, RIPK1, CEBPD, FZD6, LEF1, FZD5, MAP2K3, IL1RAPL1, PDGFD, IRAK4, FZD7, CAMK2G

IL-22 Signaling	1.32E00	2.4E-01	SOCS3, AKT2, AKT1, IL10RB, MAPK10, STAT3
Tumoricidal Function of Hepatic Natural Killer Cells	1.32E00	2.5E-01	FADD, CASP6, CASP3, BAX, CASP7, FAS
Airway Pathology in Chronic Obstructive Pulmonary Disease	1.28E00	3.33E-01	IL8, MMP2, MMP9
mTOR Signaling	1.27E00	1.42E-01	TSC1, AKT2, NRAS, RHOC, RRAS, DIRAS3, RPS6KA3, FKBP1A, RHOJ, RICTOR, EIF4G1, PPP2R5A, EIF4EBP1, VEGFA, HMOX1, AKT1, EIF4G2, EIF3B, RND3, EIF4A1, EIF3I, PRKAG2, PRKCE
Myc Mediated Apoptosis Signaling	1.18E00	1.8E-01	FADD, TP53, SHC1, AKT2, AKT1, NRAS, CASP3, RRAS, MAPK10, BAX, FAS
Bladder Cancer Signaling	1.12E00	1.63E-01	TP53, IL8, DAPK1, NRAS, RRAS, THBS1, MMP14, MMP2, FGF13, VEGFA, FGF12, CHD1, ERBB2, MMP9, RASSF1
G Beta Gamma Signaling	1.12E00	1.28E-01	ADCY2, AKT2, NRAS, RRAS, GNAI1, GNG5, SHC1, GNAI3, AKT1, PRKAG2, CAV1, PRKCE, CAV2, GNAL, GNG12
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	1.09E00	1.33E-01	SFRP2, MMP14, CSNK1A1, FZD1, TRADD, JUN, AKT1, PPP3CB, SMO, TRAF5, NFKBIB, FZD2, PPP3CA, TNFRSF11B, BMP1, ITGB1, AKT2, SPP1, ITGA2, ITGA5, IKBKE, ITGA3, TCF3, ITGB3, COL1A1, MAPK10, FZD6, FZD5, LEF1, MAP2K3, IL1RAPL1, FZD7
Androgen Signaling	1.09E00	1.25E-01	CALR, CDK7, GNAI1, GTF2E2, GNG5, SHC1, GNAI3, HSPA4, POLR2L, TGFB1I1, JUN, POLR2E, PRKAG2, POLR2H, PRKCE, DNAJB1, GNG12, GNAL
Endothelin-1 Signaling	1.09E00	1.37E-01	PDIA3, CASP4, CASP6, SHC1, HMOX1, JUN, PLCE1, PLA2G5, CASQ1, CASP1, PLCB1, PRKCE, ECE1, ADCY2, NRAS, CASP3, RRAS, GNAI1, PLA2G6, GNAI3, ARAF, CASP2, MAPK10, CASP7, GNAL
Regulation of eIF4 and p70S6K Signaling	1.08E00	1.29E-01	ITGB1, AKT2, NRAS, RRAS, ITGA2, ITGA5, ITGA3, EIF4G1, PPP2R5A, EIF4EBP1, SHC1, AKT1, EIF4G2, EIF3B, EIF4A1, EIF3I, ITGA4
Oncostatin M Signaling	1.08E00	2E-01	SHC1, NRAS, RRAS, OSMR, STAT3, PLAU, CHI3L1
Interferon Signaling	1.08E00	1.94E-01	PTPN2, IFNGR2, PSMB8, BAX, TAP1, BAK1, IRF1
MIF Regulation of Innate Immunity	1.06E00	1.6E-01	TP53, PLA2G6, LY96, JUN, PLA2G5, MAPK10, CD14, NFKBIB
GABA Receptor Signaling	1.05E00	1.58E-01	GABBR2, GABRG2, GABRB3, GABRG1, GABRB1, SLC6A1, GABBR1, ALDH5A1, AP2S1
IGF-1 Signaling	1.03E00	1.5E-01	SOCS3, IGFBP4, PXN, AKT2, NRAS, RRAS, SOCS2, IGFBP5, IGFBP7, STAT3, GRB10, SHC1, JUN, AKT1, IGFBP3, PRKAG2
IL-17A Signaling in Fibroblasts	1.02E00	1.75E-01	JUN, CEBPD, IL17RC, IKBKE, CEBPB, NFKBIZ, NFKBIB
Cardiac Hypertrophy Signaling	1.01E00	1.31E-01	MYL6, PDIA3, DIRAS3, CACNA1E, AKT1, JUN, PLCE1, PPP3CB, TGFB2, PLCB1, MAPKAPK2, PPP3CA, GNG12, ADCY2, NRAS, RHOC, RRAS, GNAI1, CACNA1C, RHOJ, CACNA1A, GNG5, MYL9, GNAI3, MYL12A, RND3, MYL12B, MAPK10, PRKAG2, MAP2K3, GNAL, HSPB1
ERK/MAPK Signaling	1.01E00	1.32E-01	RAP1B, DUSP6, RAPGEF4, EIF4EBP1, ELF4, SHC1, PLA2G5, PRKCE, PPP1CA, ITGA4, ITGB1, PXN, NRAS, RRAS, ITGA2, ITGA5, STAT3, MKNK2, ITGA3, PPP2R5A, PLA2G6, PAK3, ARAF, PRKAG2, DUSP4, PAK7, HSPB1

Type II Diabetes Mellitus Signaling	1.01E00	1.12E-01	PKM2, SOCS3, AKT2, SOCS2, ACSL6, IKBKE, CEBPB, KCNJ11, TRADD, AKT1, PRKAG2, ABCC8, MAPK10, PRKCE, SLC27A3, NFKBIB, SMPD3, TNFRSF11B
Mismatch Repair in Eukaryotes	1E00	1.67E-01	PCNA, RFC4, RFC2, POLD1
Atherosclerosis Signaling	9.91E-01	1.4E-01	IL8, PDGFA, CXCR4, F3, TNFRSF14, TNFRSF12A, COL1A2, COL1A1, PLA2G6, PLA2G5, PDGFD, COL18A1, MMP9, ITGA4, COL3A1
fMLP Signaling in Neutrophils	9.9E-01	1.33E-01	NOX4, NRAS, RRAS, ARPC5, GNAI1, GNG5, GNAI3, ACTR3, PPP3CB, ARPC2, ARPC4, PRKCE, PLCB1, ARPC1A, NFKBIB, GNG12, PPP3CA
Wnt/β-catenin Signaling	9.74E-01	1.44E-01	SFRP2, FRAT1, ILK, CSNK1A1, FZD1, AKT1, JUN, TGFB2, SMO, RUVBL2, FZD2, ACVR1C, TP53, AKT2, CSNK1G2, HDAC1, TCF3, PPP2R5A, CDH2, CD44, FZD6, BTRC, LEF1, FZD5, FZD7
Glutamate Receptor Signaling	9.71E-01	1.45E-01	GRM5, SLC1A6, SLC1A4, GRID1, GRIN2C, GRIA2, GRIP1, GRIK2, GNG5, GRIN3A
Reelin Signaling in Neurons	9.65E-01	1.59E-01	ITGB1, MAPK8IP2, ITGA2, ITGA5, ITGA3, RELN, CDK5R1, ITGB3, APBB1, AKT1, MAPK10, MAPT, ITGA4
Ovarian Cancer Signaling	9.59E-01	1.41E-01	TP53, AKT2, NRAS, RRAS, MMP2, FZD1, TCF3, VEGFA, AKT1, FZD6, PRKAG2, SMO, CD44, BRCA2, LEF1, FZD5, BRCA1, FZD2, MMP9, FZD7
IL-17 Signaling	9.53E-01	1.62E-01	IL8, AKT2, AKT1, JUN, NRAS, RRAS, TIMP1, MAPK10, IL17RC, MAP2K3, CEBPB, MAPKAPK2
VEGF Signaling	9.47E-01	1.41E-01	PXN, AKT2, NRAS, RRAS, ACTB, ACTA2, ACTG1, VEGFA, SHC1, AKT1, ACTN4, VCL, ACTG2, ACTN1
Angiopoietin Signaling	9.42E-01	1.49E-01	AKT2, ANGPT2, TNIP1, AKT1, NRAS, PAK3, RRAS, IKBKE, PAK7, NFKBIB, BIRC5
Protein Kinase A Signaling	9.36E-01	1.28E-01	RAP1B, AKAP12, AKAP8, MYL6, PDIA3, AKAP11, GYS1, PLCE1, PPP3CB, FLNA, HLA-B, SMO, TGFB2, PRKCE, PLCB1, RYR1, PPP1CA, NFKBIB, PPP3CA, GNG12, VASP, PXN, PDE2A, ADCY2, GNAI1, PYGL, AKAP6, TCF3, GNG5, MYL9, GNAI3, MYL12A, ADD3, FLNC, MYL12B, KDELR2, KDELR3, PRKAG2, PDE8B, LEF1, KDELR1, CAMK2G
CREB Signaling in Neurons	9.31E-01	1.24E-01	PDIA3, SHC1, POLR2L, AKT1, PLCE1, GRID1, GRIN2C, PRKCE, PLCB1, POLR2H, GRIK2, GNG12, ADCY2, AKT2, NRAS, RRAS, GNAI1, GRIA2, GNG5, GRM5, GNAI3, POLR2E, PRKAG2, GNAL, CAMK2G
NRF2-mediated Oxidative Stress Response	9.07E-01	1.35E-01	PPIB, ACTA2, GCLC, DNAJC10, CLPP, HMOX1, SOD2, AKT1, JUN, ABCC1, PRKCE, ACTG2, GCLM, DNAJB1, TXN, GSTK1, NRAS, RRAS, ACTB, MAFF, ACTG1, ERP29, MGST2, DNAJB11, MAP2K3, EIF2AK3
Synaptic Long Term Potentiation	8.92E-01	1.4E-01	RAP1B, NRAS, RRAS, PPP1R1A, GRIA2, CACNA1C, GRIN3A, GRM5, PPP3CB, GRIN2C, PRKAG2, PRKCE, PLCB1, PPP1CA, PPP3CA, CAMK2G
Semaphorin Signaling in Neurons	8.86E-01	1.73E-01	ITGB1, RND1, RND3, PAK3, RHOC, DIRAS3, RHOJ, PAK7, NRP1
Endometrial Cancer Signaling	8.86E-01	1.58E-01	TP53, AKT2, AKT1, NRAS, RRAS, CTNNA1, ILK, LEF1, ERBB2
Colorectal Cancer Metastasis Signaling	8.82E-01	1.28E-01	DIRAS3, MMP14, FZD1, BIRC5, VEGFA, JUN, AKT1, SMO, TGFB2, FZD2, GNG12, TP53, ADCY2, AKT2, NRAS, CASP3, RHOC, RRAS, ADRBK2, RHOJ, MMP2, STAT3, BAX, TCF3, GNG5, RND3, MAPK10, PRKAG2, FZD6, FZD5, LEF1, MMP9, FZD7
Circadian Rhythm Signaling	8.77E-01	1.71E-01	PER3, GRIN2C, VIPR2, BHLHE40, CRY2, GRIN3A
Hypoxia Signaling in the Cardiovascular System	8.74E-01	1.62E-01	VEGFA, TP53, P4HB, HSP90B1, AKT1, JUN, UBE2S, NFKBIB, LDHA, UBE2J2, UBE2I
Hepatic Cholestasis	8.42E-01	1.16E-01	IL8, ADCY2, TJP2, SLC4A2, SLCO1A2, IKBKE, HSD3B7, IRAK1, LY96, JUN, ABCC1, PRKAG2, MAPK10, CD14, PRKCE, IL1RAPL1, NFKBIB, ABCC3, IRAK4, TNFRSF11B
IL-10 Signaling	8.41E-01	1.41E-01	HMOX1, SOCS3, JUN, FCGR2A, IL10RB, CD14, IKBKE, MAP2K3, STAT3, IL1RAPL1, NFKBIB

EIF2 Signaling	8.14E-01	1.29E-01	AKT2, NRAS, RRAS, PPP1R15A, EIF4G1, SHC1, AKT1, EIF3B, EIF4G2, EIF4A1, EIF3I, PPP1CA, EIF2AK3
TREM1 Signaling	8.13E-01	1.36E-01	ITGB1, IL8, TREM1, AKT2, AKT1, CASP1, ITGA5, STAT3, IRAK1
IL-1 Signaling	8.05E-01	1.31E-01	ADCY2, GNAI1, IKBKE, GNG5, IRAK1, GNAI3, JUN, PRKAG2, MAPK10, MAP2K3, NFKBIB, GNAL, IRAK4, GNG12
Cardiac β-adrenergic Signaling	8.02E-01	1.24E-01	AKAP12, PDE2A, ADCY2, AKAP8, PPP1R1A, ADRBK2, SLC8A3, CACNA1C, AKAP6, PPP2R5A, CACNA1A, GNG5, AKAP11, CACNA1E, PKIB, PRKAG2, PDE8B, PPP1CA, GNG12
HER-2 Signaling in Breast Cancer	7.97E-01	1.48E-01	ITGB1, TP53, TSC1, AKT2, AKT1, NRAS, RRAS, CDK6, PRKCE, MMP2, ERBB2, ITGB3
Role of MAPK Signaling in the Pathogenesis of Influenza	7.94E-01	1.54E-01	PLA2G6, AKT2, AKT1, NRAS, CASP3, RRAS, PLA2G5, MAPK10, MAP2K3, BAX
Antiproliferative Role of TOB in T Cell Signaling	7.8E-01	1.92E-01	CCNA2, TGFB2, TWSG1, CDK2, SKP2
Tight Junction Signaling	7.75E-01	1.34E-01	AKT2, TJP2, MYL6, ACTB, ACTA2, CTNNA1, CSDA, ACTG1, PPP2R5A, CPSF4, MYL9, F11R, JUN, AKT1, TGFB2, PRKAG2, MYH9, ACTG2, VCL, SPTAN1, VASP, TNFRSF11B
CD40 Signaling	7.62E-01	1.43E-01	TANK, JUN, MAPK10, TNFAIP3, IKBKE, MAP2K3, STAT3, TRAF5, MAPKAPK2, NFKBIB
Chronic Myeloid Leukemia Signaling	7.53E-01	1.33E-01	TP53, AKT2, NRAS, HDAC4, RRAS, HDAC1, CDK6, IKBKE, HDAC5, E2F6, AKT1, HDAC3, HDAC11, TGFB2
SAPK/JNK Signaling	7.53E-01	1.37E-01	TP53, NRAS, RRAS, MAPK8IP2, GNG5, FADD, SHC1, TRADD, JUN, RIPK1, GADD45A, MAPK10, FCER1G, DUSP4
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	7.41E-01	1.35E-01	JUN, NRAS, PPP3CB, RRAS, BCL10, VAV3, MAPK10, TGFB2, IKBKE, MALT1, NFKBIB, PPP3CA
α-Adrenergic Signaling	7.34E-01	1.24E-01	ADCY2, NRAS, RRAS, SLC8A3, GNAI1, PYGL, GNG5, GNAI3, GYS1, HLA-B, PRKAG2, PRKCE, GNG12
Lipid Antigen Presentation by CD1	7.32E-01	1.74E-01	CALR, ARF6, PDIA3, FCER1G
DNA Methylation and Transcriptional Repression Signaling	7.32E-01	1.74E-01	HDAC1, SAP30, DNMT1, SAP18
Coagulation System	6.97E-01	1.58E-01	PROS1, SERPINA1, PLAU, SERPINE1, F3, PLAT
Nucleotide Excision Repair Pathway	6.97E-01	1.71E-01	POLR2L, RPA3, ERCC1, POLR2E, CDK7, POLR2H
Prolactin Signaling	6.94E-01	1.38E-01	SHC1, SOCS3, JUN, NRAS, RRAS, SOCS2, PRKCE, NMI, STAT3, CEBPB, IRF1
DNA Double-Strand Break Repair by Homologous Recombination	6.84E-01	1.76E-01	LIG1, BRCA2, BRCA1
Aryl Hydrocarbon Receptor Signaling	6.79E-01	1.19E-01	TP53, CDK6, BAX, FAS, CHEK1, CCNA2, HSP90B1, JUN, MGST2, TGFB2, ALDH16A1, CHEK2, ALDH5A1, ALDH6A1, CDK2, AHR, MCM7, HSPB1, GSTK1
CD27 Signaling in Lymphocytes	6.62E-01	1.4E-01	SIVA1, JUN, CASP3, MAPK10, IKBKE, MAP2K3, TRAF5, NFKBIB
Glucocorticoid Receptor Signaling	6.43E-01	1.15E-01	HSPA6, GTF2E2, CD163, HSPA5, HSPA4, SHC1, HSP90B1, POLR2L, AKT1, JUN, HSPA1L, PPP3CB, ANXA1, TGFB2, POLR2H, SERPINE1, NFKBIB, PPP3CA, TAF12, IL8, SRA1, AKT2, NRAS, RRAS, CDK7, IKBKE, STAT3, CEBPB, POLR2E, MAPK10, PRKAG2, NR3C2, PLAU, UBE2I
TNFR2 Signaling	6.42E-01	1.47E-01	TANK, JUN, TNFAIP3, IKBKE, NFKBIB

PI3K Signaling in B Lymphocytes	6.4E-01	1.26E-01	AKT2, ATF3, NRAS, PDIA3, RRAS, IKBKE, MALT1, PLEKHA4, AKT1, JUN, PLCE1, PPP3CB, BCL10, VAV3, PLCB1, NFKBIB, PPP3CA, CAMK2G
Maturity Onset Diabetes of Young (MODY) Signaling	6.29E-01	1.29E-01	CACNA1E, GAPDH, CACNA1C, CACNA1A
CD28 Signaling in T Helper Cells	6.18E-01	1.21E-01	AKT2, ARPC5, IKBKE, MALT1, ACTR3, AKT1, JUN, PPP3CB, BCL10, ARPC2, ARPC4, FCER1G, MAPK10, ARPC1A, NFKBIB, PPP3CA
P2Y Purigenic Receptor Signaling Pathway	6.18E-01	1.19E-01	ADCY2, AKT2, NRAS, PDIA3, RRAS, GNAI1, GNG5, ITGB3, GNAI3, AKT1, JUN, PLCE1, PRKAG2, PLCB1, PRKCE, GNG12
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	6.18E-01	1.36E-01	FADD, CASP6, HLA-A, CASP3, HLA-E, HLA-B, FCER1G, HLA-G, CASP7, FAS, HLA-C
Intrinsic Prothrombin Activation Pathway	6.02E-01	1.47E-01	COL1A2, COL1A1, PROS1, COL18A1, COL3A1
Role of Tissue Factor in Cancer	5.97E-01	1.32E-01	TP53, ITGB1, IL8, P4HB, AKT2, NRAS, CASP3, RRAS, RPS6KA3, ITGA3, F3, ITGB3, VEGFA, AKT1, PLCB1
Melatonin Signaling	5.95E-01	1.3E-01	GNAI3, PLCE1, ARAF, PDIA3, GNAI1, PRKAG2, PRKCE, PLCB1, MAP2K3, CAMK2G
Acute Myeloid Leukemia Signaling	5.94E-01	1.34E-01	AKT2, AKT1, NRAS, ARAF, RRAS, KIT, LEF1, MAP2K3, STAT3, TCF3, EIF4EBP1
Graft-versus-Host Disease Signaling	5.79E-01	1.4E-01	HLA-A, HLA-E, HLA-B, FCER1G, HLA-G, FAS, HLA-C
GM-CSF Signaling	5.72E-01	1.34E-01	SHC1, AKT2, AKT1, NRAS, PPP3CB, RRAS, STAT3, PPP3CA, CAMK2G
VDR/RXR Activation	5.71E-01	1.36E-01	SERPINB1, SPP1, GADD45A, PDGFA, IGFBP3, TGFB2, PRKCE, CD14, IGFBP5, HES1, CEBPB
Basal Cell Carcinoma Signaling	5.71E-01	1.37E-01	TP53, SMO, FZD6, LEF1, FZD5, FZD1, TCF3, FZD2, FZD7, BMP1
Granzyme B Signaling	5.68E-01	1.88E-01	CASP3, LMNB2, LMNB1
Glioma Signaling	5.53E-01	1.16E-01	TP53, AKT2, NRAS, RRAS, PDGFA, CDK6, CDKN2C, SHC1, E2F6, AKT1, PRKCE, PDGFD, CAMK2G
Autoimmune Thyroid Disease Signaling	5.5E-01	1.15E-01	HLA-A, HLA-E, HLA-B, FCER1G, HLA-G, FAS, HLA-C
Nitric Oxide Signaling in the Cardiovascular System	5.49E-01	1.1E-01	VEGFA, PDE2A, HSP90B1, AKT2, CACNA1E, AKT1, CAV1, PRKAG2, CACNA1C, CHRM1, CACNA1A
IL-3 Signaling	5.47E-01	1.35E-01	SHC1, AKT2, AKT1, JUN, NRAS, PPP3CB, RRAS, PRKCE, STAT3, PPP3CA
Small Cell Lung Cancer Signaling	5.47E-01	1.12E-01	TP53, AKT2, AKT1, CKS1B, CDK6, IKBKE, TRAF5, NFKBIB, CDK2, SKP2
Activation of IRF by Cytosolic Pattern Recognition Receptors	5.47E-01	1.25E-01	TANK, FADD, JUN, RIPK1, PPIB, MAPK10, IKBKE, IRF3, NFKBIB
Aldosterone Signaling in Epithelial Cells	5.44E-01	1.18E-01	ICMT, PDIA3, DNAJC12, HSPA6, DNAJC10, DNAJC2, HSPA5, HSPA12A, HSPA4, HSP90B1, PLCE1, HSPA1L, DNAJB11, ACCN2, PLCB1, PRKCE, NR3C2, DNAJB1, HSPB1, AHCY
Insulin Receptor Signaling	5.31E-01	1.22E-01	FOXO4, SOCS3, TSC1, AKT2, NRAS, RRAS, TRIP10, ACLY, GRB10, EIF4EBP1, SHC1, AKT1, GYS1, ACCN2, HLA-B, PRKAG2, PPP1CA
Thrombin Signaling	5.07E-01	1.17E-01	AKT2, ADCY2, NRAS, MYL6, RHOC, PDIA3, RRAS, DIRAS3, GNAI1, RHOJ, GNG5, MYL9, GNAI3, SHC1, MYL12A, PLCE1, AKT1, RND3, MYL12B, PLCB1, PRKCE, GNG12, GNAL, CAMK2G
IL-17A Signaling in Gastric Cells	5.03E-01	1.6E-01	IL8, JUN, MAPK10, IL17RC

Role of JAK family kinases in IL-6-type Cytokine Signaling	5.03E-01	1.48E-01	SOCS3, MAPK10, OSMR, STAT3
MIF-mediated Glucocorticoid Regulation	4.97E-01	1.19E-01	PLA2G6, LY96, PLA2G5, CD14, NFKBIB
Complement System	4.97E-01	1.43E-01	C1R, SERPING1, C5AR1, C1S, CFI
Human Embryonic Stem Cell Pluripotency	4.83E-01	1.1E-01	AKT2, PDGFA, SMAD7, FZD1, TCF3, NOG, AKT1, FZD6, TGFB2, SMO, S1PR1, LEF1, FZD5, PDGFD, FZD2, BMP1, FZD7
Crosstalk between Dendritic Cells and Natural Killer Cells	4.73E-01	1.24E-01	HLA-A, HLA-E, ICAM3, ACTB, ACTA2, HLA-B, ACTG2, HLA-G, ACTG1, FAS, HLA-C, CAMK2G
14-3-3-mediated Signaling	4.72E-01	1.25E-01	TSC1, AKT2, NRAS, RRAS, PDIA3, BAX, TUBB, AKT1, JUN, PLCE1, MAPK10, PRKCE, PLCB1, TUBA1C, MAPT
Melanoma Signaling	4.67E-01	1.3E-01	TP53, AKT2, AKT1, NRAS, RRAS, CHD1
PTEN Signaling	4.67E-01	1.13E-01	ITGB1, FOXO4, AKT2, NRAS, CASP3, RRAS, ITGA2, ILK, ITGA5, IKBKE, ITGA3, SHC1, AKT1, ITGA4
Erythropoietin Signaling	4.57E-01	1.15E-01	SHC1, SOCS3, AKT2, AKT1, JUN, NRAS, RRAS, PRKCE, NFKBIB
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	4.5E-01	1.23E-01	TP53, AKT2, NRAS, RRAS, STAT3, FZD1, SHC1, AKT1, FZD6, SMO, FZD5, FZD2, BMP1, FZD7
Cholecystokinin/Gastrin-mediated Signaling	4.44E-01	1.23E-01	PXN, NRAS, RRAS, RHOC, DIRAS3, RHOJ, SHC1, JUN, RND3, MAPK10, PRKCE, PLCB1, MAP2K3
Role of JAK2 in Hormone-like Cytokine Signaling	4.37E-01	1.35E-01	SH2B3, SHC1, SOCS3, SOCS2, STAT3
CCR5 Signaling in Macrophages	4.17E-01	9.57E-02	GNAI3, JUN, MAPK10, GNAI1, FCER1G, PRKCE, GNG5, GNG12, FAS
Role of RIG1-like Receptors in Antiviral Innate Immunity	4.16E-01	1.22E-01	TANK, FADD, RIPK1, IKBKE, IRF3, NFKBIB
Thyroid Cancer Signaling	4.16E-01	1.3E-01	TP53, SHC1, NRAS, RRAS, LEF1, TCF3
JAK/Stat Signaling	4.09E-01	1.25E-01	SHC1, SOCS3, AKT2, AKT1, NRAS, RRAS, SOCS2, STAT3
Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza	4.06E-01	1.23E-01	CSNK1G2, CSNK1A1, SMO, FZD6, LEF1, FZD5, FZD1, TCF3, FZD2, FZD7
PPAR Signaling	4.05E-01	1.13E-01	SHC1, SRA1, HSP90B1, JUN, NRAS, RRAS, PDGFA, IKBKE, IL1RAPL1, PDGFD, NFKBIB, TNFRSF11B
Chemokine Signaling	3.98E-01	1.22E-01	GNAI3, JUN, NRAS, RRAS, CXCR4, GNAI1, PLCB1, PPP1CA, CAMK2G
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	3.93E-01	1.33E-01	TP53, CASP6, SPP1, SH3GLB1, BRCA1, PHB
Role of NFAT in Regulation of the Immune Response	3.9E-01	1.05E-01	AKT2, NRAS, FCGR2A, RRAS, CSNK1G2, CSNK1A1, GNAI1, IKBKE, GNG5, RCAN1, GNAI3, AKT1, JUN, PPP3CB, FCER1G, PLCB1, NFKBIB, GNG12, PPP3CA, GNAL, ORAI1
4-1BB Signaling in T Lymphocytes	3.75E-01	1.18E-01	JUN, MAPK10, IKBKE, NFKBIB
Sonic Hedgehog Signaling	3.75E-01	1.21E-01	SMO, PRKAG2, CDK1, CCNB1
GNRH Signaling	3.71E-01	1.03E-01	ADCY2, NRAS, RRAS, GNAI1, DNM3, GNAI3, JUN, PAK3, PRKAG2, MAPK10, PRKCE, PLCB1, MAP2K3, PAK7, CAMK2G
p38 MAPK Signaling	3.68E-01	1.23E-01	MKNK2, FAS, IRAK1, FADD, PLA2G6, TRADD, PLA2G5, TGFB2, MAP2K3, IL1RAPL1, MAPKAPK2, IRAK4, HSPB1
AMPK Signaling	3.66E-01	9.64E-02	TSC1, AKT2, CHRNA9, PFKL, PPP2R5A, EIF4EBP1, AKT1, GYS1, PFKFB4, PRKAG2, CPT2, HLA-B, MAP2K3, ACACA, AK4, AK2

April Mediated Signaling	3.6E-01	1.16E-01	JUN, MAPK10, IKBKE, TRAF5, NFKBIB
OX40 Signaling Pathway	3.56E-01	1.11E-01	JUN, HLA-A, HLA-E, MAPK10, HLA-B, FCER1G, TRAF5, HLA-G, NFKBIB, HLA-C
Role of IL-17A in Psoriasis	3.53E-01	1.54E-01	IL8, IL17RC
LPS-stimulated MAPK Signaling	3.46E-01	1.1E-01	JUN, NRAS, RRAS, MAPK10, PRKCE, CD14, IKBKE, MAP2K3, NFKBIB
Leptin Signaling in Obesity	3.46E-01	1.1E-01	SOCS3, ADCY2, AKT2, AKT1, PLCE1, PDIA3, PRKAG2, PLCB1, STAT3
HGF Signaling	3.45E-01	1.14E-01	RAP1B, ELF4, PXN, AKT2, AKT1, JUN, NRAS, RRAS, MAPK10, PRKCE, STAT3, CDK2
Relaxin Signaling	3.41E-01	1.01E-01	RAP1B, PDE2A, ADCY2, AKT2, GNAI1, GNG5, VEGFA, GNAI3, AKT1, JUN, PRKAG2, PDE8B, NFKBIB, GNG12, MMP9, GNAL
Non-Small Cell Lung Cancer Signaling	3.36E-01	1.01E-01	TP53, AKT2, AKT1, NRAS, RRAS, CDK6, ERBB2, RASSF1
Polyamine Regulation in Colon Cancer	3.34E-01	1.03E-01	SAT1, PSME2, OAZ1
Neuropathic Pain Signaling In Dorsal Horn Neurons	3.32E-01	1.11E-01	GRM5, KCNN3, KCNN2, PLCE1, PDIA3, GRIN2C, GRIA2, PRKAG2, PRKCE, PLCB1, CAMK2G, GRIN3A
TGF-β Signaling	3.26E-01	1.12E-01	JUN, NRAS, RRAS, HDAC1, SMAD7, TGFB2, MAP2K3, SERPINE1, ACVR1C, TGIF1
B Cell Activating Factor Signaling	3.17E-01	1.11E-01	JUN, MAPK10, IKBKE, TRAF5, NFKBIB
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	3.13E-01	9.63E-02	RAP1B, AKT2, RHOC, DIRAS3, IFNGR2, RHOJ, IKBKE, PPP2R5A, IRF1, AKT1, JUN, RND3, CYBA, MAPK10, PRKCE, NFKBIB, PPP1CA, TNFRSF11B
Toll-like Receptor Signaling	3.11E-01	1.09E-01	LY96, JUN, CD14, MAP2K3, IRAK4, IRAK1
p70S6K Signaling	3.01E-01	1.08E-01	AKT2, NRAS, RRAS, PDIA3, GNAI1, PPP2R5A, SHC1, GNAI3, AKT1, PLCE1, PRKCE, PLCB1, MAPT, BCAP31
FcγRIIB Signaling in B Lymphocytes	2.97E-01	8.47E-02	SHC1, AKT1, NRAS, RRAS, MAPK10
Assembly of RNA Polymerase II Complex	2.94E-01	1.07E-01	TAF12, POLR2L, POLR2E, CDK7, POLR2H, GTF2E2
HIF1α Signaling	2.94E-01	1.11E-01	VEGFA, TP53, AKT2, AKT1, JUN, NRAS, RRAS, MMP14, MAPK10, MMP2, LDHA, MMP9
Role of JAK1, JAK2 and TYK2 in Interferon Signaling	2.81E-01	1.11E-01	PTPN2, IFNGR2, STAT3
G Protein Signaling Mediated by Tubby	2.8E-01	9.76E-02	PLCB1, TUB, GNG5, GNG12
Neurotrophin/TRK Signaling	2.75E-01	1.04E-01	SHC1, AKT1, JUN, NRAS, RRAS, SPRY1, SPRY2, MAP2K3
Ceramide Signaling	2.73E-01	1.03E-01	AKT2, AKT1, JUN, NRAS, RRAS, S1PR1, SMPD3, PPP2R5A, TNFRSF11B
Renal Cell Carcinoma Signaling	2.61E-01	1.08E-01	VEGFA, AKT2, AKT1, JUN, NRAS, PAK3, RRAS, PAK7
IL-9 Signaling	2.6E-01	1E-01	SOCS3, SOCS2, BCL3, STAT3
Extrinsic Prothrombin Activation Pathway	2.54E-01	1E-01	PROS1, F3
Cell Cycle Regulation by BTG Family Proteins	2.41E-01	1.11E-01	E2F6, PRMT1, CDK2, PPP2R5A

Appendix Table A2. Pearson correlation of integrin pathway genes with IGFBP2

Integrin Pathway													
Gene	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**
ZYX	0.784	ITGB3	0.513	MAP2K2	0.312	DNAJC15	0.18	GNAS	0.053	PAK1	-0.114	MRAS	-0.304
RHOJ	0.751	UBE2I	0.504	CDC34	0.308	HSP90AA1	0.18	ITGA8	0.05	TSPAN1	-0.116	GIT1	-0.312
MYL12A	0.748	RALA	0.496	GNA15	0.305	FYB	0.176	DNAJC9	0.033	MYL2	-0.122	PNCK	-0.312
HSPA4	0.746	AKT2	0.495	GNB4	0.296	ITGAM	0.175	DNAJC19	0.03	GNAQ	-0.132	CAMK2A	-0.319
GNG5	0.732	UBE2J2	0.493	ASAP1	0.292	PIK3R5	0.171	GSK3B	0.027	UBE2L3	-0.133	UBE2R2	-0.321
SHC1	0.723	ARF5	0.488	RHOD	0.291	UBE2V2	0.171	MAP2K1	0.027	ITGB6	-0.136	SNCA	-0.332
PFN1	0.714	UBE2F	0.488	WIPF1	0.288	MYLK	0.162	GNA14	0.022	CRKL	-0.142	PTK2	-0.334
TSPAN4	0.713	PXN	0.482	ITGAE	0.287	DNAJC8	0.161	UBE2G1	0.015	FYN	-0.142	PIK3C2G	-0.337
ITGA7	0.699	UBE2A	0.48	UBE2D4	0.285	RHOF	0.161	ROCK1	0.013	MYL7	-0.151	DOCK1	-0.338
ITGA5	0.697	AKT1	0.473	ACTC1	0.283	PTGES3	0.152	UBE3B	0.012	PIK3R4	-0.157	GNG13	-0.338
HSP90B1	0.696	ARF1	0.468	GNB1	0.282	NEDD4L	0.151	PIK3R2	0.01	DNM1	-0.158	DCTN1	-0.342
GNG12	0.69	UBE2D3	0.462	RHOT2	0.281	PIK3CD	0.151	DNAJB5	-0.002	RHOV	-0.162	CAMK4	-0.368
DIRAS3	0.675	SMURF1	0.454	PAK2	0.278	ACTA1	0.15	MYL5	-0.003	GNAT1	-0.163	GNB3	-0.371
ITGB1	0.671	CTTN	0.451	GNB2L1	0.271	GRB2	0.142	MAP3K11	-0.006	RHOT1	-0.164	AIP	-0.404
VAV3	0.67	NFE2L2	0.45	GNB1L	0.267	GNG7	0.141	RRAS2	-0.008	SRC	-0.164	HSPB8	-0.404
RAP2B	0.668	ITGB2	0.449	PIK3R3	0.267	DNAJC17	0.138	BCAR3	-0.009	DNAJC5B	-0.165	MAPK8	-0.417
HSPA5	0.664	RHOQ	0.449	GSN	0.25	MAPK1	0.137	ARHGAP26	-0.01	PTPN11	-0.167	UBE2E2	-0.429
DNAJB11	0.661	ITGB8	0.448	DNAJB9	0.247	DNAJC13	0.132	GNAT2	-0.01	MYLK2	-0.17	DNAJC6	-0.432
MYL12B	0.66	RAP1A	0.44	PIK3C3	0.246	TSPAN2	0.129	HSP90AB1	-0.01	ARF3	-0.172	NR3C1	-0.434
DNAJB1	0.659	GNG11	0.429	TSPAN6	0.241	UBE2E1	0.126	DNAJC7	-0.011	GNB5	-0.172	PTEN	-0.444
PPP1CA	0.654	DNAJC14	0.424	VAV1	0.239	DNAJC4	0.125	CALML5	-0.021	ITGAD	-0.172	ARHGAP5	-0.45
ITGA3	0.649	UBA1	0.42	RALB	0.229	RAPGEF1	0.125	DNAJB7	-0.021	CAMK2B	-0.173	PIK3R1	-0.451
RHOC	0.648	NCK2	0.414	CAMK1	0.227	WASL	0.124	DNAJA4	-0.026	HSPA2	-0.176	GNAZ	-0.455
ARF4	0.64	PARVA	0.411	ITGA6	0.217	ITGA10	0.121	UBE2B	-0.028	PAK6	-0.184	GAB2	-0.457
ACTA2	0.639	LCP2	0.409	UBE2L6	0.213	PPP1CC	0.121	PICK1	-0.031	PIK3CB	-0.185	RND2	-0.475
ACTG1	0.615	ITGAV	0.396	UBE2N	0.213	DNAJC16	0.12	HTT	-0.037	TSPAN3	-0.194	TNK2	-0.49
DNAJC10	0.608	GNA12	0.395	PIK3R6	0.203	RAF1	0.12	RHOB	-0.037	DNAJB2	-0.204	SH3GL3	-0.498
PARVB	0.603	PPP1CB	0.395	RHOG	0.202	RHOH	0.12	HSPA14	-0.066	AKT3	-0.21	PFN2	-0.502
ILK	0.6	VAV2	0.389	DNAJC11	0.201	UBE2J1	0.119	RHOU	-0.07	GLS	-0.213	PPARA	-0.511
ARF6	0.599	ITGB5	0.386	HSPA8	0.199	HIP1	0.118	DNAJB8	-0.075	DNAJB13	-0.214	DNAJB12	-0.531
GNAI3	0.599	CTSD	0.382	UBE2E3	0.199	WAS	0.112	FNBP1	-0.076	ATM	-0.216	GNAI1	-0.535
UBE2S	0.597	CRK	0.38	ITGB4	0.198	UBE2G2	0.103	TSPAN5	-0.08	PPP1R12B	-0.22	TSPAN7	-0.536

ACTB	0.59	DNAJC3	0.373	ATP5B	0.197	ITGA9	0.099	DNAJB14	-0.081	MYLK3	-0.223	HSPA1L	-0.538
NRAS	0.587	TLN1	0.373	GNA11	0.197	DNAJC1	0.094	PIK3CA	-0.081	KRAS	-0.227	WASF1	-0.542
ACTG2	0.581	UBE2M	0.372	DNM2	0.196	PLCG2	0.088	ITGB7	-0.087	GNG3	-0.242	PAK3	-0.569
HSPA6	0.578	UBD	0.371	PIK3CG	0.196	DNAJA1	0.086	PIK3C2B	-0.087	ITGA2B	-0.246	GNAL	-0.585
VASP	0.572	IRS2	0.37	HRAS	0.195	ITGAX	0.086	UBE2D1	-0.087	GRM7	-0.255	PAK7	-0.604
ITGA4	0.568	LIMS1	0.366	SMURF2	0.195	BRAF	0.085	GNA13	-0.09	BIRC6	-0.266	RAP2A	-0.605
RAP1B	0.565	SDHB	0.356	NCK1	0.193	GNG2	0.085	DNAJB4	-0.092	CAMK1G	-0.267	CAMK2G	-0.612
RND3	0.557	RHOA	0.347	PFN4	0.191	UBE3A	0.085	ILKAP	-0.092	BCAR1	-0.268	DNM3	-0.66
RRAS	0.541	DNAJA3	0.339	ABL1	0.187	DNAJC5	0.081	OPN1SW	-0.095	DNAJC18	-0.268	DNAJC12	-0.705
VCL	0.531	GNAI2	0.337	ITGA11	0.187	GRB7	0.071	DNAJA2	-0.097	GNG4	-0.284		
ITGA2	0.529	UBE2H	0.33	NEDD4	0.186	AR	0.069	PPP1R12A	-0.097	MAP2K4	-0.284		
CAV1	0.526	HSPA9	0.324	ITGAL	0.185	UBE2D2	0.066	CAMK2D	-0.103	DNAJC5G	-0.288		
AHR	0.523	PAK4	0.32	UBE2Q1	0.183	PIK3C2A	0.055	DNAJC21	-0.105	TLN2	-0.289		

Appendix Table A3. Pearson correlation of ILK pathway genes with IGFBP2

ILK Pathway														
Gene	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**	**Pearson**	**Gene**
VEGFA	0.837	UBE2I	0.504	RHOD	0.291	PPP2CA	0.165	FLNB	0.03	CAMK2D	-0.103	ITGA2B	-0.246	HSPA1L
FLNA	0.754	AKT2	0.495	ITGAE	0.287	DNAJC8	0.161	GSK3B	0.027	DNAJC21	-0.105	NACA	-0.251	CAMK2G
RHOJ	0.751	UBE2J2	0.493	UBE2D4	0.285	RHOF	0.161	ACTN3	0.024	TESK1	-0.105	GRM7	-0.255	PPP2R5A
HSPA4	0.746	UBE2F	0.488	ACTC1	0.283	PTGES3	0.152	PPP2R5C	0.021	TNNT3	-0.107	TNF	-0.26	DNM3
ACTN1	0.742	PXN	0.482	GNB1	0.282	NEDD4L	0.151	UBE2G1	0.015	PPP2CB	-0.111	BIRC6	-0.266	DNAJC12
GNG5	0.732	UBE2A	0.48	RHOT2	0.281	PIK3CD	0.151	UBE3B	0.012	MYH13	-0.119	CAMK1G	-0.267	RICTOR
TPM4	0.718	FLNC	0.475	HIF1A	0.279	ACTA1	0.15	MYH6	0.01	MYL2	-0.122	DNAJC18	-0.268	
ITGA7	0.699	AKT1	0.473	GNB2L1	0.271	GNG7	0.141	PIK3R2	0.01	MYH2	-0.129	MYH3	-0.271	
ITGA5	0.697	UBE2D3	0.462	GNB1L	0.267	MTOR	0.14	CCND1	0.007	IRS1	-0.13	EP300	-0.273	
HSP90B1	0.696	TCF3	0.461	PIK3R3	0.267	DNAJC17	0.138	DNAJB5	-0.002	SH2B2	-0.13	TNNT2	-0.275	
MYH9	0.695	SMURF1	0.454	ATF4	0.255	MAPK1	0.137	MYL5	-0.003	UBE2L3	-0.133	PDPK1	-0.283	
CASP3	0.692	PPP2R1B	0.451	SNAI1	0.254	GSK3A	0.136	TNNI3	-0.007	MYH4	-0.134	DNAJC5G	-0.288	
TGFB1I1	0.689	NFE2L2	0.45	GSN	0.25	DNAJC13	0.132	HSP90AB1	-0.01	ITGB6	-0.136	PPP2R3A	-0.293	
FBLIM1	0.677	ITGB2	0.449	DNAJB9	0.247	CTNNB1	0.127	DNAJC7	-0.011	ARHGEF6	-0.143	CREB3L4	-0.303	
DIRAS3	0.675	RHOQ	0.449	PIK3C3	0.246	UBE2E1	0.126	CALML5	-0.021	TNNI1	-0.143	TNNT1	-0.303	
ITGB1	0.671	ITGB8	0.448	RSU1	0.246	DNAJC4	0.125	DNAJB7	-0.021	CFL2	-0.151	PPP2R2B	-0.304	
HSPA5	0.664	MYL4	0.441	CAMK1	0.227	ITGA10	0.121	MYC	-0.024	MYL7	-0.151	PNCK	-0.312	
DNAJB11	0.661	CREB5	0.43	PTGS2	0.22	DNAJC16	0.12	DNAJA4	-0.026	PIK3R4	-0.157	CAMK2A	-0.319	
DNAJB1	0.659	GNG11	0.429	ITGA6	0.217	RHOH	0.12	UBE2B	-0.028	DNM1	-0.158	UBE2R2	-0.321	
ACTN4	0.654	DNAJC14	0.424	UBE2L6	0.213	UBE2J1	0.119	BCL9	-0.031	MYH10	-0.16	MAP2K6	-0.324	
MYL6	0.651	UBA1	0.42	UBE2N	0.213	HIP1	0.118	PICK1	-0.031	MYH8	-0.16	SNCA	-0.332	
ITGA3	0.649	NCK2	0.414	PIK3R6	0.203	ATF2	0.115	TNNI2	-0.033	RHOV	-0.162	PTK2	-0.334	
RHOC	0.648	MUC1	0.411	PPM1J	0.202	HNF1A	0.108	PPAP2B	-0.035	RHOT1	-0.164	PIK3C2G	-0.337	
ACTA2	0.639	PARVA	0.411	RHOG	0.202	VEGFC	0.106	TAF4	-0.036	SRC	-0.164	DOCK1	-0.338	
MYL9	0.637	ITGAV	0.396	DNAJC11	0.201	UBE2G2	0.103	HTT	-0.037	DNAJC5B	-0.165	DCTN1	-0.342	
JUN	0.636	ITGB5	0.386	HSPA8	0.199	ITGA9	0.099	RHOB	-0.037	PGF	-0.167	CAMK4	-0.368	
TPM2	0.627	CTSD	0.382	UBE2E3	0.199	TNNC1	0.099	MYH1	-0.043	PPM1L	-0.167	GNB3	-0.371	
ACTG1	0.615	DNAJC3	0.373	ITGB4	0.198	DNAJC1	0.094	FIGF	-0.045	PTPN11	-0.167	MYH7B	-0.39	
MMP9	0.609	UBE2M	0.372	ATP5B	0.197	CREB3	0.09	TCF4	-0.048	GNB5	-0.172	AIP	-0.404	
DNAJC10	0.608	UBD	0.371	DNM2	0.196	DNAJA1	0.086	ACTN2	-0.063	ITGAD	-0.172	HSPB8	-0.404	
FN1	0.603	IRS2	0.37	PIK3CG	0.196	ITGAX	0.086	HSPA14	-0.066	CAMK2B	-0.173	PPP2R2C	-0.415	
PARVB	0.603	FERMT2	0.367	SMURF2	0.195	GNG2	0.085	RHOU	-0.07	HSPA2	-0.176	TCF7L2	-0.424	
SNAI2	0.601	SDHB	0.356	PPP2R2A	0.194	UBE3A	0.085	MYH11	-0.071	PIK3CB	-0.185	UBE2E2	-0.429	
ILK	0.6	FOS	0.353	ITGA11	0.187	DNAJC5	0.081	DNAJB8	-0.075	PPP2R5B	-0.201	DNAJC6	-0.432	

UBE2S	0.597	PDGFC	0.351	NEDD4	0.186	MYL6B	0.079	FNBP1	-0.076	TCF7L1	-0.201	NR3C1	-0.434
ACTB	0.59	RHOA	0.347	ITGAL	0.185	AR	0.069	DNAJB14	-0.081	DNAJB2	-0.204	MYH7	-0.438
ACTG2	0.581	DNAJA3	0.339	UBE2Q1	0.183	UBE2D2	0.066	PIK3CA	-0.081	FKBP4	-0.204	PTEN	-0.444
HSPA6	0.578	TPM1	0.335	DNAJC15	0.18	NDEL1	0.061	TNNC2	-0.085	RPS6KA4	-0.205	PIK3R1	-0.451
ITGA4	0.568	NOS2	0.334	HSP90AA1	0.18	PPP2R5D	0.059	ITGB7	-0.087	AKT3	-0.21	MYH14	-0.453
CHD1	0.562	UBE2H	0.33	PPP2R4	0.178	PIK3C2A	0.055	PIK3C2B	-0.087	PROK1	-0.211	GAB2	-0.457
RND3	0.557	HSPA9	0.324	VEGFB	0.177	ITGA8	0.05	UBE2D1	-0.087	GLS	-0.213	RND2	-0.475
LEF1	0.545	PPP2R1A	0.323	ITGAM	0.175	TBP	0.045	TAF9B	-0.088	DNAJB13	-0.214	CREBBP	-0.486
VCL	0.531	CFL1	0.311	PPP2R5E	0.175	PPP2R3B	0.042	DNAJB4	-0.092	ATM	-0.216	SH3GL3	-0.498
ITGA2	0.529	CDC34	0.308	PIK3R5	0.171	DSP	0.038	ILKAP	-0.092	IRS4	-0.216	PPARA	-0.511
AHR	0.523	CREB1	0.304	UBE2V2	0.171	DNAJC9	0.033	DNAJA2	-0.097	MYL3	-0.231	BMP2	-0.521
ITGB3	0.513	GNB4	0.296	MAF	0.165	DNAJC19	0.03	PPP1R12A	-0.097	MYL1	-0.236	DNAJB12	-0.531

Appendix Table A4. Altered signaling pathways resulting from disruption of IGFBP2-integrin binding.

Pathways	-log (p-value)	Ratio	Molecules
Virus Entry via Endocytic Pathways	9.02E00	2.3E-01	RAC2,FLNB,ITSN1,RRAS,PIK3R1,ACTA2,HRAS,PLCG1,ITGA5,ITGB8,ACTG1,PRKCZ,ITGB3,HLA-A,FLNA,MRAS,HLA-B,PRKCE,ACTG2,CXADR,ITGB5,HLA-C,PRKCA
Molecular Mechanisms of Cancer	8.57E00	1.29E-01	PRKACB,RAF1,GAB2,RAC2,DIRAS3,PIK3R1,SUV39H1,HRAS,CCND1,PTK2,CAMK2D,MRAS,HIPK2,FZD2,ARHGEF4,RRAS,CDKN2D,ARHGEF17,RHOJ,BCL2L1,RHOQ,ARHGEF16,FZD5,LEF1,FNBP1,CDK2,TCF4,HIF1A,MAP3K5,PRKCZ,ARHGEF19,SHC1,HHAT,FANCD2,BBC3,RHOU,TGFB2,PRKCE,ARHGEF2,PRKCA,MAPK8,GNAI1,FADD,PRKAR2B,FOXO1,CDKN1A,BMP6,WNT5A,CTNND1
ILK Signaling	7.67E00	1.66E-01	MYH10,FLNB,FN1,PIK3R1,DIRAS3,ACTA2,PDPK1,HIF1A,ITGB8,CCND1,PTK2,FLNA,KRT18,RHOU,IRS2,ACTG2,DSP,ITGB5,MUC1,FBLIM1,MAPK8,VEGFC,RHOJ,ACTG1,ITGB3,RHOQ,SNAI2,MYH9,LEF1,RPS6KA5,PPP2R1B,FNBP1
IGF-1 Signaling	7.45E00	2.06E-01	PRKACB,IGFBP6,SOCS3,RAF1,RRAS,PIK3R1,SOCS2,MAPK8,HRAS,PDPK1,IGFBP5,IGFBP7,PRKCZ,IGFBP2,GRB10,PTK2,SHC1,PRKAR2B,FOXO1,IGFBP3,MRAS,IRS2
Integrin Signaling	6.9E00	1.52E-01	RAC2,RAF1,PIK3R1,DIRAS3,ACTA2,HRAS,PPP1CB,ITGB8,MYLK,PTK2,SHC1,MRAS,RHOU,ACTG2,TSPAN4,ITGB5,RRAS,ASAP1,MAPK8,ITGA5,PLCG1,RHOJ,ACTG1,ITGB3,WIPF1,RHOQ,ARF5,TLN2,ARF3,TSPAN6,ITGA7,FNBP1
PTEN Signaling	6.74E00	1.77E-01	RAC2,RAF1,RRAS,FGFR1,PIK3R1,HRAS,PDPK1,ITGA5,FOXG1,CCND1,PRKCZ,PTK2,FGFR3,BCL2L1,SHC1,FOXO1,CDKN1A,KDR,PDGFRA,MRAS,FGFRL1,MAGI3
HER-2 Signaling in Breast Cancer	6.56E00	2.22E-01	RRAS,PIK3R1,NRG1 (includes EG:112400),HRAS,PLCG1,ITGB8,MAP3K5,CCND1,PRKCZ,ITGB3,FOXO1,CDKN1A,MRAS,PRKCE,ERBB2,PARD3,ITGB5,PRKCA
Thrombin Signaling	6.29E00	1.45E-01	RAF1,F2R,PIK3R1,DIRAS3,HRAS,PDPK1,GNG13,PPP1CB,PRKCZ,MYLK,PTK2,SHC1,CAMK2D,GNG11,MRAS,RHOU,PRKCE,ARHGEF2,GNG4,PRKCA,ARHGEF4,RRAS,GNAI1,PLCG1,RHOJ,ITPR1,RHOQ,ARHGEF16,ITPR3,FNBP1
IL-8 Signaling	6.18E00	1.45E-01	RAC2,RAF1,NAPEPLD,PIK3R1,DIRAS3,HRAS,GNG13,CCND1,PRKCZ,PTK2,HMOX1,GNG11,RHOU,MRAS,PRKCE,GNG4,PRKCA,ANGPT1,RRAS,MAPK8,GNAI1,VEGFC,RHOJ,BCL2L1,RHOQ,ARAF,KDR,FNBP1
Agrin Interactions at Neuromuscular Junction	5.94E00	2.32E-01	RAC2,NRG2,RRAS,ACTA2,NRG1 (includes EG:112400),MAPK8,HRAS,ITGA5,ACTG1,ITGB3,PTK2,MRAS,UTRN,ACTG2,ERBB2,AGRN
α-Adrenergic Signaling	5.66E00	1.7E-01	PRKACB,RAF1,RRAS,GNAI1,GNG13,HRAS,PLCG1,ITPR1,PRKCZ,GNG11,PRKAR2B,ITPR3,MRAS,HLA-B,PRKCE,SLC8A1,GNG4,PRKCA
Glioma Invasiveness Signaling	5.51E00	2.33E-01	F2R,RRAS,DIRAS3,PIK3R1,HRAS,RHOJ,ITGB3,PTK2,TIMP4,RHOQ,MRAS,RHOU,FNBP1,ITGB5
Caveolar-mediated Endocytosis Signaling	5.43E00	1.88E-01	FLNB,ARCN1,ITSN1,ACTA2,ITGA5,ITGB8,ACTG1,ITGB3,HLA-A,FLNA,HLA-B,ACTG2,ITGA7,ITGB5,HLA-C,PRKCA
Cholecystokinin/Gastrin-mediated Signaling	5.36E00	1.79E-01	RAF1,RRAS,DIRAS3,MAPK8,HRAS,RHOJ,ITPR1,PRKCZ,PTK2,SHC1,RHOQ,ITPR3,CREM,MRAS,RHOU,PRKCE,MEF2C,FNBP1,PRKCA

Role of NFAT in Cardiac Hypertrophy	5.29E00	1.3E-01	PRKACB,RAF1,LIF,PIK3R1,HRAS,GNG13,IL6,PRKCZ,SHC1,CAMK2D,GNG11,MRAS,TGFB2,PRKCE,GNG4,PRKCA,RRAS,MAPK8,GNAI1,PLCG1,ITPR1,RCAN1,PRKAR2B,ITPR3,MEF2C,SLC8A1,IL11
Breast Cancer Regulation by Stathmin1	5.18E00	1.33E-01	PRKACB,RAF1,PIK3R1,GNG13,HRAS,PPP1CB,PRKCZ,ARHGEF19,SHC1,CAMK2D,GNG11,MRAS,PRKCE,ARHGEF2,GNG4,PRKCA,ARHGEF4,RRAS,GNAI1,TUBA4A,ARHGEF17,ITPR1,PRKAR2B,ARHGEF16,ITPR3,CDKN1A,PPP2R1B,CDK2
VEGF Signaling	5.09E00	1.72E-01	RAF1,RRAS,PIK3R1,ACTA2,HRAS,VEGFC,PLCG1,HIF1A,ACTG1,PTK2,SHC1,BCL2L1,FOXO1,KDR,MRAS,ACTG2,PRKCA
Axonal Guidance Signaling	5.09E00	1.04E-01	SLIT3,PRKACB,RAF1,RAC2,ITSN1,EPHB2,PIK3R1,HRAS,GNG13,ROBO1,PRKCZ,PTK2,SHC1,GNG11,EFNB1,ABLIM3,MRAS,PRKCE,ERBB2,EFNB3,SEMA3B,FZD2,RTN4R,GNG4,PRKCA,NGEF,NRP2,RRAS,TUBA4A,GNAI1,ITGA5,VEGFC,SLIT2,DPYSL5,EPHA3,SEMA3A,WIPF1,PRKAR2B,ADAM12,PLXNB1,FZD5,BMP6,EPHA2,WNT5A,NRP1
Glioblastoma Multiforme Signaling	4.99E00	1.4E-01	RAF1,RRAS,PIK3R1,DIRAS3,HRAS,PLCG1,RHOJ,ITPR1,CCND1,SHC1,RHOQ,FOXO1,CDKN1A,ITPR3,PDGFRA,MRAS,RHOU,LEF1,FZD5,FZD2,FNBP1,CDK2,WNT5A
G Beta Gamma Signaling	4.96E00	1.45E-01	PRKACB,RAF1,RRAS,GNAI1,GNG13,HRAS,PDPK1,PLCG1,PRKCZ,SHC1,GNG11,PRKAR2B,MRAS,PRKCE,CAV2,GNG4,PRKCA
Phospholipase C Signaling	4.55E00	1.15E-01	RAF1,NAPEPLD,DIRAS3,HRAS,GNG13,PPP1CB,PRKCZ,ARHGEF19,SHC1,HMOX1,GNG11,AHNAK,MRAS,RHOU,PRKCE,ARHGEF2,GNG4,PRKCA,ARHGEF4,RRAS,ITGA5,PLCG1,ARHGEF17,RHOJ,ITPR1,RHOQ,ARHGEF16,ITPR3,MEF2C,FNBP1
Semaphorin Signaling in Neurons	4.52E00	2.31E-01	PTK2,MET,SEMA3A,RHOQ,DPYSL3,DIRAS3,PLXNB1,RHOU,RHOJ,DPYSL5,FNBP1,NRP1
VDR/RXR Activation	4.48E00	1.85E-01	IGFBP6,SPP1,IGFBP5,HES1 (includes EG:15205),PRKCZ,COL13A1,FOXO1,RUNX2,CDKN1A,TGFB2,CEBPA,IGFBP3,PRKCE,SEMA3B,PRKCA
Germ Cell-Sertoli Cell Junction Signaling	4.42E00	1.38E-01	RAC2,RRAS,PIK3R1,DIRAS3,ACTA2,PVRL3,TUBA4A,MAPK8,HRAS,PDPK1,RHOJ,MAP3K5,GSN,ACTG1,PTK2,RHOQ,MRAS,RHOU,TGFB2,JUP,ACTG2,FNBP1,CTNND1
Bladder Cancer Signaling	4.36E00	1.74E-01	RAF1,MMP3,RRAS,THBS1,SUV39H1,MMP14,VEGFC,HRAS,CCND1,FGF13,FGFR3,FGF12,CDKN1A,MRAS,RPS6KA5,ERBB2
HGF Signaling	4.33E00	1.62E-01	RAF1,RRAS,PIK3R1,MAPK8,HRAS,PLCG1,IL6,MAP3K5,CCND1,PRKCZ,PTK2,MET,CDKN1A,MRAS,PRKCE,CDK2,PRKCA
Renin-Angiotensin Signaling	4.31E00	1.43E-01	PRKACB,RAF1,RRAS,PIK3R1,MAPK8,HRAS,PLCG1,ITPR1,PRKCZ,PTK2,SHC1,PRKAR2B,CCL2,SHC2,ITPR3,MRAS,PRKCE,PRKCA
Insulin Receptor Signaling	4.27E00	1.43E-01	PRKACB,RAF1,SOCS3,RRAS,PIK3R1,MAPK8,PPP1CB,HRAS,PDPK1,LIPE,PRKCZ,GRB10,SHC1,PRKAR2B,RHOQ,FOXO1,MRAS,ACCN2,HLA-B,IRS2
Thrombopoietin Signaling	4.26E00	1.9E-01	GAB2,SHC1,RAF1,RRAS,PIK3R1,MRAS,PRKCE,PLCG1,HRAS,IRS2,PRKCZ,PRKCA
Prolactin Signaling	4.21E00	1.75E-01	RAF1,SOCS3,RRAS,PIK3R1,SOCS2,PLCG1,PDPK1,HRAS,PRKCZ,SHC1,MRAS,PRKCE,NMI,PRKCA
Non-Small Cell Lung Cancer Signaling	4.14E00	1.65E-01	RAF1,RRAS,PIK3R1,SUV39H1,PLCG1,PDPK1,HRAS,ITPR1,CCND1,ITPR3,MRAS,ERBB2,PRKCA
FGF Signaling	4.09E00	1.67E-01	RAF1,PIK3R1,FGFR1,MAPK8,HRAS,PLCG1,MAP3K5,ITPR1,FGF13,FGFR3,MET,FGF12,RPS6KA5,FGFRL1,PRKCA
Glioma Signaling	4.01E00	1.43E-01	RAF1,RRAS,SUV39H1,PIK3R1,CDKN2D,HRAS,PLCG1,CCND1,PRKCZ,SHC1,CAMK2D,CDKN1A,PDGFRA,MRAS,PRKCE,PRKCA

Macropinocytosis Signaling	3.93E00	1.71E-01	RRAS,PIK3R1,PLCG1,ITGA5,HRAS,ITGB8,PRKCZ,ITGB3,MET,MRAS,PRKCE,ITGB5,PRKCA
Colorectal Cancer Metastasis Signaling	3.91E00	1.12E-01	PRKACB,TCF4,MMP3,PIK3R1,DIRAS3,MMP14,HRAS,GNG13,IL6,CCND1,GNG11,MRAS,TGFB2,RHOU,FZD2,GNG4,PTGER4,RRAS,MAPK8,VEGFC,RHOJ,BCL2L1,PRKAR2B,RHOQ,FZD5,LEF1,TCF7L2,FNBP1,WNT5A
Neuregulin Signaling	3.85E00	1.47E-01	RAF1,NRG2,RRAS,PIK3R1,NRG1 (includes EG:112400),HRAS,PLCG1,ITGA5,PDPK1,PRKCZ,SHC1,MRAS,PRKCE,ERBB2,PRKCA
Ephrin Receptor Signaling	3.81E00	1.15E-01	RAC2,RAF1,NGEF,ITSN1,ANGPT1,EPHB2,RRAS,PTPN13,GNAI1,GNG13,HRAS,VEGFC,ITGA5,EPHA3,PTK2,SHC1,WIPF1,GNG11,EFNB1,MRAS,EFNB3,GNG4,EPHA2
CXCR4 Signaling	3.72E00	1.24E-01	RAF1,RRAS,PIK3R1,DIRAS3,MAPK8,GNAI1,GNG13,HRAS,RHOJ,ITPR1,PRKCZ,PTK2,GNG11,RHOQ,ITPR3,MRAS,RHOU,PRKCE,GNG4,FNBP1,PRKCA
Prostate Cancer Signaling	3.7E00	1.44E-01	RAF1,RRAS,SUV39H1,PIK3R1,PDPK1,HRAS,CCND1,FOXO1,CDKN1A,MRAS,NKX3-1,LEF1,CDK2,GSTP1
Actin Cytoskeleton Signaling	3.61E00	1.09E-01	RAC2,RAF1,MYH10,FN1,F2R,PIK3R1,ACTA2,PPP1CB,HRAS,SSH1,FGF13,MYLK,PTK2,SHC1,FGF12,MRAS,ACTG2,ARHGEF4,RRAS,RDX,ITGA5,GSN,ACTG1,TIAM2,MYH9,PIP4K2A
mTOR Signaling	3.57E00	1.14E-01	NAPEPLD,DDIT4,RRAS,PIK3R1,DIRAS3,HRAS,PDPK1,FKBP1A,VEGFC,RHOJ,HIF1A,RPS4Y1,PRKCZ,HMOX1,RHOQ,MRAS,RHOU,PRKCE,RPS4Y2,RPS6KA5,PPP2R1B,AKT1S1,FNBP1,PRKCA
CCR3 Signaling in Eosinophils	3.55E00	1.35E-01	RAF1,RRAS,PIK3R1,GNAI1,PPP1CB,GNG13,HRAS,ITPR1,PRKCZ,MYLK,GNG11,ITPR3,MRAS,PRKCE,CCL26,GNG4,PRKCA
Erythropoietin Signaling	3.4E00	1.54E-01	SHC1,RAF1,SOCS3,RRAS,PIK3R1,MRAS,PRKCE,PLCG1,PDPK1,HRAS,PRKCZ,PRKCA
Acute Myeloid Leukemia Signaling	3.37E00	1.59E-01	RAF1,TCF4,RRAS,PIK3R1,HRAS,CCND1,ARAF,CSF2RA,CEBPA,MRAS,LEF1,JUP,TCF7L2
FAK Signaling	3.37E00	1.37E-01	RAF1,ASAP1,RRAS,PIK3R1,ACTA2,HRAS,PLCG1,ITGA5,PDPK1,ACTG1,PTK2,TLN2,MRAS,ACTG2
PI3K/AKT Signaling	3.33E00	1.21E-01	GAB2,RAF1,RRAS,PIK3R1,HRAS,PDPK1,ITGA5,MAP3K5,CCND1,PRKCZ,SHC1,BCL2L1,FOXO1,CDKN1A,MRAS,HLA-B,PPP2R1B
Chemokine Signaling	3.28E00	1.64E-01	PTK2,RAF1,CAMK2D,CCL2,RRAS,MAPK8,MRAS,GNAI1,PLCG1,HRAS,PPP1CB,PRKCA
Tight Junction Signaling	3.18E00	1.22E-01	PRKACB,MYH10,TJP2,RAB13,ACTA2,PVRL3,CSDA,SYMPK,ACTG1,PRKCZ,MYLK,F11R,PRKAR2B,MPP5,CEBPA,TGFB2,MYH9,ARHGEF2,ACTG2,PPP2R1B
Leukocyte Extravasation Signaling	3.18E00	1.15E-01	RAC2,MMP3,PIK3R1,MMP14,ACTA2,RDX,GNAI1,MAPK8,ARHGAP4,THY1,PLCG1,ACTG1,PRKCZ,PTK2,F11R,TIMP4,WIPF1,SIPA1,PRKCE,ARHGAP12,ACTG2,PRKCA,CTNND1
GNRH Signaling	3.12E00	1.16E-01	PRKACB,RAF1,RRAS,MAPK8,GNAI1,HRAS,DNM3,ITPR1,MAP3K5,PRKCZ,PTK2,CAMK2D,PRKAR2B,ITPR3,MRAS,PRKCE,PRKCA
Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis	3.06E00	1.04E-01	TCF4,MMP3,MMP14,PIK3R1,IL6,MAP3K5,RUNX2,TRAF5,FZD2,ADAMTS4,CTSK,SPP1,MAPK8,ITGA5,GSN,ITGB3,FOXO1,LEF1,FZD5,SFRP1,DKK1,BMP6,TCF7L2,WNT5A,IL11
Protein Kinase A Signaling	3.03E00	9.76E-02	PRKACB,AKAP12,RAF1,MYH10,FLNB,TCF4,LIPE,GNG13,PPP1CB,PRKCZ,MYLK,PTK2,CAMK2D,GNG11,HHAT,FLNA,TGFB2,HLA-B,PRKCE,GNG4,HIST1H1B,PRKCA,H1F0,GNAI1,PLCG1,ITPR1,ANAPC4,PRKAR2B,CREM,ITPR3,LEF1,TCF7L2
Biosynthesis of Steroids	3.03E00	4.96E-02	SQLE,FDFT1,DHCR7,IDI1,HMGCR,SC5DL

Regulation of Actin-based Motility by Rho	3E00	1.43E-01	RAC2,DIRAS3,ACTA2,PPP1CB,RHOJ,GSN,MYLK,WIPF1,RHOQ,RHOU,ACTG2,PIP4K2A,FNBP1
EGF Signaling	2.97E00	1.73E-01	SHC1,RAF1,PIK3R1,ITPR3,MAPK8,PLCG1,HRAS,ITPR1,PRKCA
Cardiac Hypertrophy Signaling	2.95E00	1.02E-01	PRKACB,RAF1,PIK3R1,DIRAS3,GNG13,HRAS,IL6,MAP3K5,GNG11,TGFB2,RHOU,MRAS,GNG4,ADRA1B,MAPKAPK3,RRAS,GNAI1,MAPK8,PLCG1, CACNA1C,RHOJ,PRKAR2B,RHOQ,MEF2C,FNBP1
P2Y Purigenic Receptor Signaling Pathway	2.92E00	1.17E-01	PRKACB,RAF1,RRAS,PIK3R1,GNAI1,GNG13,HRAS,PLCG1,PRKCZ,ITGB3,GNG11,PRKAR2B,MRAS,PRKCE,GNG4,PRKCA
Factors Promoting Cardiogenesis in Vertebrates	2.86E00	1.37E-01	TCF4,PRKCZ,TGFB2,PRKCE,LEF1,FZD5,MEF2C,DKK1,BMP6,FZD2,TCF7L2,CDK2,PRKCA
Endothelin-1 Signaling	2.85E00	1.09E-01	RAF1,NAPEPLD,RRAS,PIK3R1,GNAI1,MAPK8,HRAS,PLCG1,CASP4,ITPR1,PRKCZ,SHC1,HMOX1,ARAF,SHC2,ITPR3,MRAS,PRKCE,CASP5,PRKCA
Human Embryonic Stem Cell Pluripotency	2.81E00	1.1E-01	TCF4,S1PR2,PIK3R1,FGFR1,PDPK1,SOX2,FGFR3,TGFB2,MRAS,PDGFRA,LEF1,FZD5,FGFRL1,BMP6,FZD2,TCF7L2,WNT5A
Growth Hormone Signaling	2.78E00	1.47E-01	SOCS3,PIK3R1,SOCS2,CEBPA,IGFBP3,PRKCE,PLCG1,PDPK1,RPS6KA5,PRKCZ,PRKCA
NRF2-mediated Oxidative Stress Response	2.78E00	1.09E-01	RAF1,PRDX1,RRAS,PIK3R1,GSTA4,ACTA2,HSPB8,MAPK8,HRAS,MAP3K5,JUNB,ACTG1,PRKCZ,GSR,HMOX1,MRAS,PRKCE,CDC34,ACTG2,GSTP1,P RKCA
Neuroprotective Role of THOP1 in Alzheimer's Disease	2.76E00	1.48E-01	PRKACB,PRKAR2B,HLA-A,HLA-E,HLA-B,HLA-G,HLA-C,HLA-F
Antigen Presentation Pathway	2.76E00	1.86E-01	PSMB9,HLA-A,HLA-E,HLA-B,HLA-G,TAP1,HLA-C,HLA-F
Crosstalk between Dendritic Cells and Natural Killer Cells	2.72E00	1.35E-01	ACTA2,CD83,IL6,ACTG1,HLA-F,TLN2,CAMK2D,HLA-A,HLA-E,HLA-B,ACTG2,HLA-G,HLA-C
Clathrin-mediated Endocytosis Signaling	2.66E00	1.1E-01	MYO6,F2R,EPHB2,PIK3R1,ACTA2,GAK,VEGFC,DNM3,ITGA5,ITGB8,HIP1,ACTG1,FGF13,ITGB3,MET,FGF12,LDLRAP1,ACTG2,ITGB5
IL-3 Signaling	2.63E00	1.49E-01	GAB2,SHC1,RAF1,FOXO1,RRAS,PIK3R1,MRAS,PRKCE,HRAS,PRKCZ,PRKCA
Melanoma Signaling	2.61E00	1.74E-01	RAF1,RRAS,MITF,PIK3R1,CDKN1A,MRAS,HRAS,CCND1
Synaptic Long Term Potentiation	2.59E00	1.23E-01	PRKACB,RAF1,RRAS,PPP1CB,CACNA1C,HRAS,ITPR1,PRKCZ,PRKAR2B,CAMK2D,ITPR3,MRAS,PRKCE,PRKCA
Fcγ Receptor-mediated Phagocytosis in Macrophages and Monocytes	2.59E00	1.27E-01	GAB2,RAC2,NAPEPLD,PIK3R1,ACTA2,PLCG1,ACTG1,PRKCZ,HMOX1,TLN2,PRKCE,ACTG2,PRKCA
Endometrial Cancer Signaling	2.58E00	1.58E-01	RAF1,RRAS,PIK3R1,MRAS,PDPK1,HRAS,LEF1,ERBB2,CCND1
GM-CSF Signaling	2.57E00	1.49E-01	SHC1,BCL2L1,RAF1,CAMK2D,RRAS,CSF2RA,PIK3R1,MRAS,HRAS,CCND1

14-3-3-mediated Signaling	2.57E00	1.25E-01	RAF1,RRAS,PIK3R1,MAPK8,TUBA4A,HRAS,PLCG1,MAP3K5,PRKCZ,FOXO1,MRAS,PRKCE,GFAP,AKT1S1,PRKCA
Oncostatin M Signaling	2.55E00	2E-01	SHC1,RAF1,MMP3,RRAS,MT2A,MRAS,HRAS
NF-κB Activation by Viruses	2.53E00	1.34E-01	RAF1,RRAS,PIK3R1,MRAS,PRKCE,ITGA5,HRAS,ITGB5,PRKCZ,PRKCA,ITGB3
Thyroid Cancer Signaling	2.48E00	1.74E-01	SHC1,TCF4,RRAS,MRAS,HRAS,LEF1,CCND1,TCF7L2
Melanocyte Development and Pigmentation Signaling	2.47E00	1.3E-01	PRKACB,SHC1,RAF1,PRKAR2B,MC1R,RRAS,MITF,PIK3R1,MRAS,PLCG1,HRAS,RPS6KA5
fMLP Signaling in Neutrophils	2.44E00	1.09E-01	RAF1,RRAS,PIK3R1,GNAI1,GNG13,HRAS,ITPR1,PRKCZ,GNG11,ITPR3,MRAS,PRKCE,GNG4,PRKCA
p70S6K Signaling	2.42E00	1.15E-01	RAF1,F2R,EEF2,RRAS,PIK3R1,GNAI1,HRAS,PLCG1,PDPK1,PRKCZ,SHC1,MRAS,PRKCE,PPP2R1B,PRKCA
Ovarian Cancer Signaling	2.41E00	1.13E-01	PRKACB,RAF1,TCF4,RRAS,SUV39H1,PIK3R1,HRAS,VEGFC,CCND1,PRKAR2B,MRAS,LEF1,FZD5,FZD2,TCF7L2,WNT5A
ERK/MAPK Signaling	2.32E00	9.8E-02	PRKACB,RAC2,RAF1,RRAS,PIK3R1,PPP1CB,HRAS,PLCG1,ITGA5,PTK2,SHC1,PRKAR2B,TLN2,ARAF,DUSP1,MRAS,PRKCE,RPS6KA5,PPP2R1B,PRKCA
CREB Signaling in Neurons	2.31E00	9.41E-02	PRKACB,RAF1,RRAS,PIK3R1,GNAI1,GNG13,HRAS,PLCG1,ITPR1,PRKCZ,SHC1,GNG11,CAMK2D,PRKAR2B,ITPR3,MRAS,PRKCE,GNG4,PRKCA
PAK Signaling	2.3E00	1.12E-01	PTK2,MYLK,SHC1,RAF1,RRAS,PIK3R1,MAPK8,MRAS,PDGFRA,ITGA5,HRAS,EPHA3
Role of Macrophages, Fibroblasts and Endothelial Cells in Rheumatoid Arthritis	2.3E00	8.71E-02	SOCS3,RAF1,TCF4,FN1,MMP3,PIK3R1,HRAS,IL6,CCND1,CEBPG,PRKCZ,CAMK2D,CCL2,MRAS,CEBPA,PRKCE,TRAF5,FZD2,PRKCA,ADAMTS4,RRAS,PLCG1,VEGFC,LEF1,FZD5,DKK1,SFRP1,TCF7L2,WNT5A
Androgen Signaling	2.3E00	9.72E-02	PRKACB,GNAI1,GNG13,CCND1,PRKCZ,SHC1,HSPA4,GNG11,PRKAR2B,MRAS,PRKCE,TAF2,GNG4,PRKCA
CCR5 Signaling in Macrophages	2.23E00	1.06E-01	GNG11,MAPK8,MRAS,GNAI1,PRKCE,PLCG1,GNG13,GNG4,PRKCZ,PRKCA
Melatonin Signaling	2.23E00	1.3E-01	PRKACB,RAF1,PRKAR2B,CAMK2D,ARAF,GNAI1,PRKCE,PLCG1,PRKCZ,PRKCA
Neurotrophin/TRK Signaling	2.23E00	1.3E-01	SHC1,RAF1,RRAS,PIK3R1,MAPK8,MRAS,PLCG1,PDPK1,HRAS,MAP3K5
Hepatic Fibrosis / Hepatic Stellate Cell Activation	2.17E00	1.09E-01	MYH10,FN1,FGFR1,ACTA2,VEGFC,IGFBP5,IL6,MET,LY96,CCL2,KDR,IGFBP3,PDGFRA,TGFB2,LAMA1,MYH9
Mechanisms of Viral Exit from Host Cells	2.14E00	1.56E-01	CHMP2B,ACTA2,PRKCE,ACTG2,ACTG1,PRKCZ,PRKCA
PDGF Signaling	2.14E00	1.27E-01	SHC1,RAF1,RRAS,PIK3R1,MAPK8,MRAS,PDGFRA,PLCG1,HRAS,PRKCA
Chronic Myeloid Leukemia Signaling	2.12E00	1.14E-01	CTBP1,GAB2,BCL2L1,RAF1,RRAS,PIK3R1,SUV39H1,CDKN1A,MRAS,TGFB2,HRAS,CCND1
IL-15 Signaling	2.1E00	1.34E-01	PTK2,SHC1,BCL2L1,RAF1,RRAS,PIK3R1,MRAS,PLCG1,HRAS
JAK/Stat Signaling	2.1E00	1.41E-01	SHC1,RAF1,SOCS3,RRAS,PIK3R1,CDKN1A,SOCS2,MRAS,HRAS

Butanoate Metabolism	2.1E00	6.92E-02	ACAT2,C1orf93,ALDH1A3,OXCT1,LIPE,SDHC,MYO5B,HMGCS1,ELOVL6
Pancreatic Adenocarcinoma Signaling	2.1E00	1.09E-01	RAF1,NAPEPLD,PIK3R1,SUV39H1,MAPK8,VEGFC,CCND1,HMOX1,BCL2L1,CDKN1A,TGFB2,ERBB2,CDK2
FcγRIIB Signaling in B Lymphocytes	2.08E00	1.19E-01	SHC1,RRAS,PIK3R1,MAPK8,MRAS,PDPK1,HRAS
PI3K Signaling in B Lymphocytes	2.07E00	1.05E-01	RAF1,ATF3,RRAS,PIK3R1,PLCG1,PDPK1,HRAS,MALT1,PLEKHA4,ITPR1,PRKCZ,CAMK2D,ITPR3,MRAS,IRS2
LPS-stimulated MAPK Signaling	2.05E00	1.22E-01	RAF1,RRAS,PIK3R1,MAPK8,MRAS,PRKCE,HRAS,MAP3K5,PRKCZ,PRKCA
AMPK Signaling	1.98E00	8.93E-02	PRKACB,CPT1A,PIK3R1,LIPE,PDPK1,AK5,PRKAR2B,HLA-B,MRAS,AK4,IRS2,HMGCR,PPP2R1B,ADRA1B,PPAT
Sphingosine-1-phosphate Signaling	1.97E00	1.07E-01	S1PR2,DIRAS3,PIK3R1,GNAI1,PLCG1,RHOJ,CASP4,PTK2,RHOQ,PDGFRA,RHOU,CASP5,FNBP1
Apoptosis Signaling	1.88E00	1.15E-01	ENDOG,BCL2L1,RAF1,RRAS,MAPK8,MRAS,PRKCE,PLCG1,HRAS,MAP3K5,PRKCA
Ceramide Signaling	1.86E00	1.15E-01	CTSD,RAF1,S1PR2,RRAS,PIK3R1,MAPK8,MRAS,HRAS,PPP2R1B,PRKCZ
Reelin Signaling in Neurons	1.82E00	1.22E-01	PAFAH1B2,ARHGEF4,PIK3R1,CNR1,ARHGEF16,MAPK8,ITGA5,LRP8,ARHGEF2,ITGB3
Aldosterone Signaling in Epithelial Cells	1.81E00	9.41E-02	RAF1,SACS,PIK3R1,HSPB8,PDPK1,PLCG1,ITPR1,PRKCZ,HSPA4,DUSP1,ITPR3,HSPB11,ACCN2,PRKCE,PIP4K2A,PRKCA
Fc Epsilon RI Signaling	1.81E00	1.08E-01	RAF1,RAC2,RRAS,PIK3R1,MAPK8,MRAS,PRKCE,PLCG1,PDPK1,HRAS,PRKCZ,PRKCA
Graft-versus-Host Disease Signaling	1.81E00	1.4E-01	HLA-A,HLA-E,HLA-B,IL6,HLA-G,HLA-C,HLA-F
Glutamate Metabolism	1.8E00	8E-02	GSR,GNPNAT1,GLUL,MYO5B,PPAT,GFPT2
p53 Signaling	1.75E00	1.15E-01	BCL2L1,BBC3,THBS1,PIK3R1,SNAI2,CDKN1A,MAPK8,HIPK2,CCND1,CDK2,DRAM1
HMGB1 Signaling	1.72E00	1.1E-01	RHOQ,CCL2,RRAS,DIRAS3,PIK3R1,MAPK8,MRAS,RHOU,HRAS,RHOJ,FNBP1
Myc Mediated Apoptosis Signaling	1.71E00	1.31E-01	FADD,SHC1,RRAS,PIK3R1,MAPK8,MRAS,HRAS,PRKCZ
FLT3 Signaling in Hematopoietic Progenitor Cells	1.65E00	1.22E-01	GAB2,SHC1,RAF1,RRAS,PIK3R1,MRAS,PDPK1,HRAS,RPS6KA5
BMP signaling pathway	1.65E00	1.12E-01	PRKACB,RAF1,PRKAR2B,RRAS,RUNX2,MAPK8,MRAS,HRAS,BMP6
Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency	1.64E00	1.05E-01	SOX2,SHC1,RAF1,LIF,RRAS,PIK3R1,MRAS,HRAS,FZD5,BMP6,FZD2,WNT5A
Type I Diabetes Mellitus Signaling	1.64E00	9.92E-02	FADD,SOCS3,HLA-A,HLA-E,SOCS2,MAPK8,HLA-B,MYO5B,MAP3K5,HLA-G,HLA-C,HLA-F
Lysine Degradation	1.6E00	5.76E-02	TMLHE,PLOD2,ACAT2,C1orf93,ALDH1A3,SUV39H1,PIPOX,ELOVL6

Corticotropin Releasing Hormone Signaling	1.58E00	8.82E-02	PRKACB,RAF1,PRKAR2B,CNR1,ITPR3,GNAI1,PRKCE,PLCG1,MEF2C,ITPR1,PRKCZ,PRKCA
O-Glycan Biosynthesis	1.57E00	1.14E-01	ST6GALNAC6,GCNT1,ST3GAL1,WBSCR17,ST3GAL4
Huntington's Disease Signaling	1.57E00	8.4E-02	PIK3R1,MAPK8,GNG13,HRAS,PDPK1,DNM3,CASP4,ITPR1,UBE2S,HIP1,PRKCZ,BCL2L1,SHC1,HSPA4,CTSD,GNG11,PRKCE,GNG4,CASP5,PRKCA
Antiproliferative Role of Somatostatin Receptor 2	1.56E00	1.13E-01	GNG11,RRAS,PIK3R1,CDKN1A,MRAS,HRAS,GNG13,GNG4
Role of MAPK Signaling in the Pathogenesis of Influenza	1.56E00	1.21E-01	RAF1,CCL2,RRAS,MAPK8,MRAS,HRAS,MAP3K5,PRKCA
CDK5 Signaling	1.56E00	1.06E-01	PRKACB,LAMA5,RAF1,PRKAR2B,RRAS,LAMA1,MRAS,HRAS,PPP1CB,PPP2R1B
Synthesis and Degradation of Ketone Bodies	1.55E00	1.58E-01	ACAT2,OXCT1,HMGCS1
IL-2 Signaling	1.5E00	1.21E-01	SHC1,RAF1,RRAS,PIK3R1,MAPK8,MRAS,HRAS
Acute Phase Response Signaling	1.5E00	8.99E-02	ECSIT,RAF1,SOCS3,TCF4,FN1,RRAS,PIK3R1,SOCS2,MAPK8,HRAS,PDPK1,IL6,MAP3K5,HMOX1,SHC1,MRAS
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages	1.49E00	8.02E-02	DIRAS3,PIK3R1,MAPK8,PPP1CB,PLCG1,RHOJ,MAP3K5,PRKCZ,RHOQ,RHOU,PRKCE,PPP2R1B,FNBP1,SIRPA,PRKCA
NF-κB Signaling	1.48E00	9.09E-02	PRKACB,RRAS,RELB,PIK3R1,FGFR1,MAPK8,HRAS,MALT1,PRKCZ,TAB3,FGFR3,KDR,PDGFRA,MRAS,TRAF5,FGFRL1
TREM1 Signaling	1.46E00	1.06E-01	CCL2,LAT2,PLCG1,ITGA5,CD83,IL6,CASP5
Pentose Phosphate Pathway	1.46E00	6.1E-02	RBKS,PGD,PGM2L1,PGM1,TKTL1
Natural Killer Cell Signaling	1.43E00	9.91E-02	SHC1,RAF1,RAC2,RRAS,PIK3R1,MRAS,PRKCE,PLCG1,HRAS,PRKCZ,PRKCA
Rac Signaling	1.43E00	8.94E-02	PTK2,RAF1,RRAS,PIK3R1,MAPK8,MRAS,ITGA5,HRAS,PARD3,PIP4K2A,PRKCZ
Cellular Effects of Sildenafil (Viagra)	1.41E00	8.61E-02	PRKACB,MYH10,ACTA2,CACNA1C,PLCG1,PPP1CB,ITPR1,ACTG1,MYLK,PRKAR2B,ITPR3,MYH9,ACTG2
IL-9 Signaling	1.36E00	1.25E-01	SOCS3,PIK3R1,SOCS2,IRS2,BCL3
G Protein Signaling Mediated by Tubby	1.36E00	1.19E-01	GNG11,MRAS,PLCG1,GNG13,GNG4
SAPK/JNK Signaling	1.36E00	9.8E-02	FADD,SHC1,RAC2,GNG11,RRAS,PIK3R1,MAPK8,MRAS,HRAS,MAP3K5
MSP-RON Signaling Pathway	1.32E00	1.18E-01	CCL2,PIK3R1,ACTA2,ACTG2,ACTG1,PRKCZ
Role of JAK2 in Hormone-like Cytokine Signaling	1.31E00	1.35E-01	SHC1,SOCS3,SOCS2,IRS2,SIRPA

Glycerophospholipid Metabolism	1.31E00	6.52E-02	PPAPDC1A,HMOX1,NAPEPLD,LPCAT2,PPP2R2D,GPD2,BCHE,CHKA,PLCG1,AGPAT9,LPIN2,ELOVL6
Role of Tissue Factor in Cancer	1.3E00	9.65E-02	BCL2L1,RRAS,PIK3R1,MRAS,VEGFC,HRAS,RPS6KA5,F3,ITGB5,PRKCA,ITGB3
Cdc42 Signaling	1.3E00	8.33E-02	RAF1,MAPK8,PPP1CB,ITGA5,PRKCZ,HLA-F,MYLK,WIPF1,HLA-A,HLA-E,FNBP1L,HLA-B,PARD3,HLA-G,HLA-C
Autoimmune Thyroid Disease Signaling	1.29E00	9.84E-02	HLA-A,HLA-E,HLA-B,HLA-G,HLA-C,HLA-F
Aryl Hydrocarbon Receptor Signaling	1.28E00	8.18E-02	GSTA4,MAPK8,IL6,CCND1,CTSD,ALDH1A3,CDKN1A,TGFB2,NFIB,NRIP1,ALDH16A1,GSTP1,CDK2
IL-17 Signaling	1.26E00	1.08E-01	CCL2,MMP3,RRAS,PIK3R1,MAPK8,MRAS,HRAS,IL6
RhoA Signaling	1.26E00	9.65E-02	PTK2,MYLK,NGEF,ACTA2,ARHGAP4,RDX,ARHGAP12,PPP1CB,ACTG2,PIP4K2A,ACTG1
TR/RXR Activation	1.25E00	9.38E-02	RAB3B,AKR1C3,SREBF2,PIK3R1,THRA,BCL3,HIF1A,DIO2,NRGN
OX40 Signaling Pathway	1.23E00	9.47E-02	BCL2L1,HLA-A,HLA-E,MAPK8,HLA-B,TRAF5,HLA-G,HLA-C,HLA-F
Neuropathic Pain Signaling In Dorsal Horn Neurons	1.21E00	9.26E-02	PRKACB,PRKAR2B,CAMK2D,PIK3R1,ITPR3,PRKCE,PLCG1,ITPR1,PRKCZ,PRKCA
PPARα/RXRα Activation	1.19E00	8.06E-02	PRKACB,RAF1,RRAS,MAPK8,PLCG1,HRAS,IL6,ABCA1,SHC1,PRKAR2B,GPD2,TGFB2,MRAS,ITGB5,PRKCA
ERK5 Signaling	1.19E00	1.09E-01	LIF,RRAS,MRAS,HRAS,MEF2C,RPS6KA5,PRKCZ
Type II Diabetes Mellitus Signaling	1.17E00	6.88E-02	SOCS3,PIK3R1,SOCS2,MAPK8,PRKCE,PDPK1,IRS2,SLC27A3,MAP3K5,PRKCZ,PRKCA
RAR Activation	1.16E00	8.02E-02	PRKACB,TRIM24,PIK3R1,MAPK8,PDPK1,MAP3K5,PRKCZ,PRKAR2B,DUSP1,ALDH1A3,TGFB2,IGFBP3,PRKCE,NRIP1,PRKCA
Role of JAK1 and JAK3 in γc Cytokine Signaling	1.16E00	1.03E-01	SHC1,SOCS3,RRAS,PIK3R1,MRAS,HRAS,IRS2
Phenylalanine Metabolism	1.14E00	4.5E-02	DHCR24,PRDX5,PRDX1,ALDH1A3,ELOVL6
B Cell Receptor Signaling	1.14E00	8.33E-02	GAB2,RAF1,RAC2,RRAS,PIK3R1,MAPK8,HRAS,MALT1,MAP3K5,SHC1,BCL2L1,CAMK2D,MRAS
CNTF Signaling	1.11E00	1.09E-01	RAF1,RRAS,PIK3R1,MRAS,HRAS,RPS6KA5
Angiopoietin Signaling	1.1E00	9.46E-02	PTK2,ANGPT1,FOXO1,RRAS,PIK3R1,MRAS,HRAS
Gα12/13 Signaling	1.09E00	8.59E-02	PTK2,RAF1,F2R,RRAS,PIK3R1,MAPK8,MRAS,HRAS,MEF2C,MAP3K5,CDH11
IL-1 Signaling	1.08E00	8.41E-02	PRKACB,ECSIT,GNG11,PRKAR2B,MAPK8,MRAS,GNAI1,GNG13,GNG4
Regulation of IL-2 Expression in Activated and Anergic T Lymphocytes	1.07E00	8.99E-02	RAF1,RRAS,MAPK8,MRAS,TGFB2,PLCG1,HRAS,MALT1
Cyclins and Cell Cycle	1.07E00	8.99E-02	RAF1,SUV39H1,CDKN1A,CDKN2D,TGFB2,PPP2R1B,CCND1,CDK2

Regulation			
Nitric Oxide Signaling in the Cardiovascular System	1.07E00	8E-02	PRKACB,PRKAR2B,PIK3R1,KDR,ITPR3,CACNA1C,VEGFC,ITPR1
Role of IL-17F in Allergic Inflammatory Airway Diseases	1E00	1.09E-01	RAF1,CCL2,RPS6KA5,IL6,IL11
Regulation of eIF4 and p70S6K Signaling	9.99E-01	7.26E-02	RAF1,RRAS,PIK3R1,ITGA5,PDPK1,HRAS,EIF2C2,RPS4Y1,PRKCZ,SHC1,MRAS,RPS4Y2,PPP2R1B
Inositol Phosphate Metabolism	9.94E-01	6.67E-02	INPP4B,ARAF,PIK3R1,MTMR14,MTMR1,MAPK8,MTM1,PRKCE,PLCG1,GRK5,PIP4K2A,CDK2
Cell Cycle: G1/S Checkpoint Regulation	9.9E-01	9.84E-02	SUV39H1,CDKN1A,NRG1 (includes EG:112400),TGFB2,CCND1,CDK2
Sulfur Metabolism	9.79E-01	5.08E-02	SULT1A1,PAPSS1,CHST11
Renal Cell Carcinoma Signaling	9.67E-01	9.46E-02	MET,RAF1,RRAS,PIK3R1,MRAS,HRAS,HIF1A
Basal Cell Carcinoma Signaling	9.67E-01	9.59E-02	TCF4,LEF1,FZD5,BMP6,FZD2,TCF7L2,WNT5A
Small Cell Lung Cancer Signaling	9.43E-01	7.87E-02	PTK2,BCL2L1,PIK3R1,SUV39H1,TRAF5,CCND1,CDK2
Glycine, Serine and Threonine Metabolism	9.43E-01	4.79E-02	C1orf93,PIPOX,CHKA,PHGDH,PLCG1,TARS,ELOVL6
Xenobiotic Metabolism Signaling	9.4E-01	7.26E-02	RAF1,RRAS,PIK3R1,GSTA4,MAPK8,HRAS,IL6,MAP3K5,PRKCZ,HS6ST1,HMOX1,CAMK2D,ALDH1A3,SULT1A1,MRAS,PRKCE,CHST11,NRIP1,ALDH16A1,PPP2R1B,GSTP1,PRKCA
Synaptic Long Term Depression	9.37E-01	7.48E-02	RAF1,RRAS,ITPR3,MRAS,GNAI1,PRKCE,HRAS,ITPR1,PPP2R1B,PRKCZ,PRKCA
Estrogen Receptor Signaling	9.37E-01	8.09E-02	CTBP1,SHC1,RAF1,RRAS,MED30,MRAS,HRAS,NRIP1,MED10,TAF2,G6PC3
Protein Ubiquitination Pathway	9.24E-01	7.3E-02	PSMB9,SACS,UBE2H,HSPB8,USP54,UBE2S,TAP1,FZR1,USP31,UCHL1,ANAPC4,HSPA4,HLA-A,HSPB11,HLA-B,USP46,CDC34,USP25,HLA-C,UBE2I
Systemic Lupus Erythematosus Signaling	9.23E-01	7.02E-02	LSM14B,RRAS,PIK3R1,HRAS,PLCG1,IL6,HLA-F,HLA-A,LSM7,HLA-E,CREM,HLA-B,MRAS,HLA-G,HLA-C,PRPF38A
Glucocorticoid Receptor Signaling	9.12E-01	6.78E-02	PRKACB,RAF1,RRAS,PIK3R1,PBX1,MAPK8,HRAS,IL6,SHC1,BCL2L1,HSPA4,CCL2,DUSP1,CDKN1A,MRAS,CEBPA,TGFB2,NRIP1,TAF2,UBE2I
Role of NFAT in Regulation of the Immune Response	9E-01	7E-02	RAF1,RRAS,PIK3R1,GNAI1,GNG13,PLCG1,HRAS,ITPR1,RCAN1,GNG11,ITPR3,MRAS,MEF2C,GNG4
PKCθ Signaling in T Lymphocytes	8.96E-01	7.04E-02	RAC2,CAMK2D,RRAS,PIK3R1,MAPK8,MRAS,PLCG1,HRAS,MALT1,MAP3K5
Galactose Metabolism	8.78E-01	4.67E-02	C1orf93,PGM2L1,PGM1,GANAB,G6PC3

HIF1α Signaling	8.55E-01	8.33E-02	MMP3,RRAS,MMP14,PIK3R1,MAPK8,MRAS,VEGFC,HRAS,HIF1A
Cardiac β-adrenergic Signaling	8.53E-01	7.14E-02	PRKACB,AKAP12,GNG11,PRKAR2B,MRAS,CACNA1C,GNG13,PPP1CB,SLC8A1,PPP2R1B,GNG4
Cell Cycle Regulation by BTG Family Proteins	8.52E-01	1.11E-01	BTG2,PPP2R1B,CCND1,CDK2
Calcium-induced T Lymphocyte Apoptosis	8.35E-01	8.57E-02	ITPR3,PRKCE,PLCG1,ITPR1,PRKCZ,PRKCA
Glycolysis/Gluconeogenesis	8.32E-01	5.97E-02	ALDH1A3,PGM2L1,PGM1,ENO2,ACYP1,ACYP2,G6PC3,BPGM
Communication between Innate and Adaptive Immune Cells	8.13E-01	7.34E-02	HLA-A,HLA-E,HLA-B,CD83,IL6,HLA-G,HLA-C,HLA-F
iCOS-iCOSL Signaling in T Helper Cells	8.02E-01	7.38E-02	GAB2,SHC1,CAMK2D,PIK3R1,ITPR3,PLCG1,PDPK1,PLEKHA4,ITPR1
CD27 Signaling in Lymphocytes	7.71E-01	8.77E-02	BCL2L1,CD70,MAPK8,TRAF5,MAP3K5
Role of JAK family kinases in IL-6-type Cytokine Signaling	7.64E-01	1.11E-01	SOCS3,MAPK8,IL6
Keratan Sulfate Biosynthesis	7.47E-01	9.26E-02	HS6ST1,ST3GAL1,SULT1A1,CHST11,ST3GAL4
ATM Signaling	7.47E-01	9.26E-02	FANCD2,CDKN1A,MAPK8,CDK2,SMC1A
LPS/IL-1 Mediated Inhibition of RXR Function	7.45E-01	6.82E-02	ECSIT,CPT1A,GSTA4,MAPK8,ABCA1,HS6ST1,LY96,ALDH1A3,SULT1A1,CHST11,FABP7,SLC27A3,ALDH16A1,HMGCS1,GSTP1
Wnt/β-catenin Signaling	7.38E-01	7.47E-02	TCF4,CCND1,SOX2,TLE4,TGFB2,LEF1,FZD5,SFRP1,DKK1,PPP2R1B,FZD2,TCF7L2,WNT5A
Cytotoxic T Lymphocyte-mediated Apoptosis of Target Cells	7.32E-01	8.14E-02	FADD,HLA-A,HLA-E,HLA-B,HLA-G,HLA-C,HLA-F
Docosahexaenoic Acid (DHA) Signaling	7.32E-01	8.16E-02	BCL2L1,FOXO1,PIK3R1,PDPK1
Antiproliferative Role of TOB in T Cell Signaling	7.28E-01	1.15E-01	TGFB2,CDC34,CDK2
Valine, Leucine and Isoleucine Degradation	7.25E-01	5.56E-02	BCAT1,ACAT2,ALDH1A3,OXCT1,HMGCS1,ELOVL6
Glycosphingolipid Biosynthesis - Lactoseries	7.18E-01	7.14E-02	ST3GAL1,ST3GAL4
T Cell Receptor Signaling	7.07E-01	7.34E-02	RAF1,RRAS,PIK3R1,MAPK8,MRAS,PLCG1,HRAS,MALT1
IL-4 Signaling	7.05E-01	8.22E-02	SHC1,RRAS,PIK3R1,MRAS,HMGA1,HRAS

Lymphotoxin β Receptor Signaling	7.01E-01	8.2E-02	BCL2L1,PIK3R1,RELB,PDPK1,TRAF5
TGF-β Signaling	6.95E-01	7.87E-02	RAF1,RRAS,RUNX2,MAPK8,MRAS,TGFB2,HRAS
Glycosphingolipid Biosynthesis - Ganglioseries	6.95E-01	5.17E-02	ST3GAL1,ST3GAL4,ELOVL6
Glycosaminoglycan Degradation	6.95E-01	5.26E-02	HYAL3,SGSH,FGFRL1
Calcium Signaling	6.89E-01	6.28E-02	PRKACB,MYH10,TPM1 (includes EG:22003),TNNT1,ACTA2,ITPR1,RCAN1,PRKAR2B,CAMK2D,ITPR3,MYH9,MEF2C,SLC8A1
PXR/RXR Activation	6.85E-01	6.74E-02	PRKACB,SCD,PRKAR2B,CPT1A,FOXO1,IL6
Starch and Sucrose Metabolism	6.66E-01	3.55E-02	UCHL1,PGM2L1,PGM1,HLA-B,GANAB,G6PC3
IL-15 Production	6.63E-01	9.38E-02	PTK2,IL6,PRKCZ
Nicotinate and Nicotinamide Metabolism	6.6E-01	5.88E-02	NNMT,ARAF,NT5E,NT5M,MAPK8,PRKCE,GRK5,CDK2
Hereditary Breast Cancer Signaling	6.32E-01	6.98E-02	FANCD2,RRAS,PIK3R1,CDKN1A,MRAS,HRAS,RFC1,CCND1,FANCA
RAN Signaling	6.29E-01	8.7E-02	RCC1,TNPO1
Extrinsic Prothrombin Activation Pathway	6.29E-01	1E-01	TFPI,F3
RANK Signaling in Osteoclasts	6.28E-01	7.37E-02	RAF1,MITF,PIK3R1,MAPK8,TRAF5,MAP3K5,GSN
Mitochondrial Dysfunction	6.11E-01	5.71E-02	MT-COI,GSR,CPT1A,PRDX5,GPD2,MAPK8,SDHC,DHODH,BACE2,PINK1
Death Receptor Signaling	5.98E-01	7.69E-02	TNFRSF21,FADD,TNFRSF25,MAPK8,MAP3K5
Methane Metabolism	5.91E-01	2.99E-02	PRDX5,PRDX1
Induction of Apoptosis by HIV1	5.79E-01	7.58E-02	FADD,BCL2L1,BBC3,MAPK8,MAP3K5
Estrogen-Dependent Breast Cancer Signaling	5.79E-01	7.14E-02	RRAS,PIK3R1,MRAS,HRAS,CCND1
Leptin Signaling in Obesity	5.79E-01	7.14E-02	PRKACB,SOCS3,PRKAR2B,FOXO1,PIK3R1,PLCG1
Selenoamino Acid Metabolism	5.78E-01	4.35E-02	PAPSS1,FTSJ1,SCLY
LXR/RXR Activation	5.63E-01	6.45E-02	SCD,LY96,CCL2,IL6,HMGCR,ABCA1
Differential Regulation of Cytokine Production in Macrophages and T	5.55E-01	1.11E-01	CCL2,IL6

Helper Cells by IL-17A and IL-17F			
Parkinson's Signaling	5.55E-01	1.11E-01	UCHL1,MAPK8
Endoplasmic Reticulum Stress Pathway	5.55E-01	1.11E-01	MAPK8,MAP3K5
Mitotic Roles of Polo-Like Kinase	5.44E-01	7.69E-02	ANAPC4,PLK2,PPP2R1B,FZR1,SMC1A
Role of Wnt/GSK-3β Signaling in the Pathogenesis of Influenza	5.32E-01	7.41E-02	TCF4,LEF1,FZD5,FZD2,TCF7L2,WNT5A
Role of CHK Proteins in Cell Cycle Checkpoint Control	5.29E-01	8.57E-02	CDKN1A,RFC1,CDK2
IL-6 Signaling	5.25E-01	7E-02	SHC1,RAF1,RRAS,MAPK8,MRAS,HRAS,IL6
Granzyme A Signaling	5.22E-01	1E-01	H1F0,HIST1H1B
Ascorbate and Aldarate Metabolism	5.22E-01	2.5E-02	C1orf93,ALDH1A3
Stilbene, Coumarine and Lignin Biosynthesis	5.22E-01	2.7E-02	PRDX5,PRDX1
Inhibition of Angiogenesis by TSP1	5.06E-01	7.69E-02	THBS1,KDR,MAPK8
PPAR Signaling	4.99E-01	6.54E-02	SHC1,RAF1,RRAS,MRAS,PDGFRA,HRAS,NRIP1
Phospholipid Degradation	4.89E-01	6.12E-02	PPAPDC1A,HMOX1,NAPEPLD,PPP2R2D,PLCG1,LPIN2
Chondroitin Sulfate Biosynthesis	4.87E-01	6.15E-02	HS6ST1,SULT1A1,CHST11,DSE
Coagulation System	4.84E-01	7.89E-02	F2R,TFPI,F3
Amyloid Processing	4.7E-01	7.14E-02	PRKACB,PRKAR2B,PRKCE,BACE2
Hypoxia Signaling in the Cardiovascular System	4.64E-01	7.35E-02	UBE2H,HIF1A,CDC34,UBE2S,UBE2I
Pyruvate Metabolism	4.64E-01	3.68E-02	ACAT2,ALDH1A3,ME3,ACYP1,ACYP2
Pantothenate and CoA Biosynthesis	4.64E-01	3.17E-02	BCAT1,DPYSL3
Nitrogen Metabolism	4.63E-01	2.48E-02	CA9,ASRGL1,GLUL
Allograft Rejection Signaling	4.62E-01	6.25E-02	HLA-A,HLA-E,HLA-B,HLA-G,HLA-C,HLA-F
cAMP-mediated signaling	4.61E-01	6.39E-02	PRKACB,AKAP12,RAF1,CNR1,GNAI1,RGS4,CHRM3,CAMK2D,PRKAR2B,MC1R,DUSP1,HTR7,CREM,PTGER4

G-Protein Coupled Receptor Signaling	4.59E-01	6.04E-02	PRKACB,RAF1,F2R,PIK3R1,PTHLH,PDPK1,HRAS,CHRM3,HRH1,SHC1,CAMK2D,MC1R,HTR7,MRAS,PRKCE,FZD2,BAI2,PTGER4,ADRA1B,PRKCA,OXTR,S1PR2,RRAS,CNR1,GNAI1,RGS4,CD97,GPR64,PRKAR2B,DUSP1,FZD5,LGR4
Polyamine Regulation in Colon Cancer	4.38E-01	6.9E-02	TCF4,SAT1
Relaxin Signaling	4.34E-01	5.7E-02	PRKACB,GNG11,PRKAR2B,PIK3R1,MRAS,GNAI1,GNG13,GNG4,PRKCZ
Notch Signaling	4.24E-01	6.98E-02	NOTCH2,LFNG,HES1 (includes EG:15205)
Aminosugars Metabolism	3.96E-01	4.1E-02	GNPNAT1,C1orf93,CYB5R2,NANS,GFPT2
Role of BRCA1 in DNA Damage Response	3.92E-01	6.56E-02	FANCD2,CDKN1A,RFC1,FANCA
IL-22 Signaling	3.91E-01	8E-02	SOCS3,MAPK8
Tumoricidal Function of Hepatic Natural Killer Cells	3.91E-01	8.33E-02	ENDOG,FADD
Role of PKR in Interferon Induction and Antiviral Response	3.89E-01	6.52E-02	FADD,TRAF5,RNASEL
EIF2 Signaling	3.88E-01	5.86E-02	EIF2AK1,RAF1,RRAS,PIK3R1,PDPK1,HRAS,EIF2C2,PPP1CB,RPS4Y1,SHC1,RPL28,MRAS,RPS4Y2
p38 MAPK Signaling	3.85E-01	6.6E-02	FADD,MAPKAPK3,DUSP1,TGFB2,MEF2C,RPS6KA5,MAP3K5
Tyrosine Metabolism	3.84E-01	2.53E-02	ALDH1A3,COMT,FTSJ1,LRTOMT,ELOVL6
Purine Metabolism	3.84E-01	4.25E-02	TJP2,AMPD1,PAICS,ATP5S,AMPD2,TAP1,NME3,AK5,NT5E,MPP5,IMPDH1,NT5M,PAPSS1,MYH9,AK4,PFAS,PPAT
Nucleotide Sugars Metabolism	3.83E-01	1.52E-02	C1orf93
Assembly of RNA Polymerase I Complex	3.83E-01	7.69E-02	TAF1C
Hepatic Cholestasis	3.79E-01	5.11E-02	PRKACB,LY96,PRKAR2B,TJP2,MAPK8,PRKCE,IL6,PRKCZ,PRKCA
Glutamate Receptor Signaling	3.78E-01	5.8E-02	GNG11,GLUL,SLC38A1,HOMER3
Propanoate Metabolism	3.78E-01	3.31E-02	ACAT2,DHCR24,ALDH1A3,SLC27A3
Glycosphingolipid Biosynthesis - Globoseries	3.7E-01	4.76E-02	ST3GAL1,ST3GAL4
Glutathione Metabolism	3.52E-01	4.4E-02	GSR,PGD,GSTA4,GSTP1
Dopamine Receptor Signaling	3.49E-01	5.26E-02	PRKACB,PRKAR2B,COMT,PPP1CB,PPP2R1B
Fatty Acid Biosynthesis	3.49E-01	2.04E-02	SLC27A3

Cell Cycle Control of Chromosomal Replication	3.31E-01	6.45E-02	DBF4,CDK2
Role of Oct4 in Mammalian Embryonic Stem Cell Pluripotency	3.14E-01	6.67E-02	SOX2,SPP1,FOXA1
4-1BB Signaling in T Lymphocytes	2.97E-01	5.88E-02	MAPK8,MAP3K5
Sonic Hedgehog Signaling	2.97E-01	6.06E-02	PRKACB,PRKAR2B
CD28 Signaling in T Helper Cells	2.95E-01	5.3E-02	PIK3R1,ITPR3,MAPK8,PLCG1,PDPK1,MALT1,ITPR1
Valine, Leucine and Isoleucine Biosynthesis	2.93E-01	2.33E-02	BCAT1
Amyotrophic Lateral Sclerosis Signaling	2.92E-01	5.04E-02	BCL2L1,NEFL,PIK3R1,CACNA1C,GLUL,VEGFC
β-alanine Metabolism	2.77E-01	3.23E-02	ALDH1A3,DPYSL3,MYO5B
Sphingolipid Metabolism	2.7E-01	4.42E-02	LASS6,PPAPDC1A,PPP2R2D,UGCG,LPIN2
Taurine and Hypotaurine Metabolism	2.69E-01	2.22E-02	MYO5B
Toll-like Receptor Signaling	2.65E-01	5.45E-02	ECSIT,LY96,MAPK8
Glycerolipid Metabolism	2.6E-01	3.87E-02	PPAPDC1A,PPP2R2D,ALDH1A3,LIPE,AGPAT9,LPIN2
Histidine Metabolism	2.55E-01	2.59E-02	ALDH1A3,FTSJ1,ELOVL6
Cysteine Metabolism	2.55E-01	3.45E-02	HS6ST1,SULT1A1,CHST11
IL-10 Signaling	2.54E-01	5.13E-02	HMOX1,SOCS3,MAPK8,IL6
TWEAK Signaling	2.28E-01	5.13E-02	FADD,TNFRSF25
Interferon Signaling	2.28E-01	5.56E-02	IFIT1,TAP1
Granzyme B Signaling	2.12E-01	6.25E-02	ENDOG
Mismatch Repair in Eukaryotes	2.12E-01	4.17E-02	RFC1
Phenylalanine, Tyrosine and Tryptophan Biosynthesis	1.97E-01	1.49E-02	ENO2

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Vita

Kristen Marie Holmes was born on December 30, 1981 in Garden City, Kansas, to Jimmy and Jan Holmes. She graduated from Holly High School in Holly, Colorado in May of 2000, and pursued her bachelor's degree at West Texas A&M University in Canyon, Texas. Kristen graduated *cum laude* in December 2003 with a biology/chemistry degree. She then pursued a master's degree at West Texas A&M University under the direction of Dr. Brenda E. Rodgers where she studied the murine genotoxic effects of environmental radiation from the Chernobyl nuclear fallout. Kristen graduated with her master's in biology in August 2006 and immediately entered the University of Texas Health Science Center at Houston Graduate School of Biomedical Sciences. She completed rotations in the laboratories of Drs. Wei Zhang, Xin Lin, and Joseph McCarty and joined Dr. Zhang's group in May of 2007. Kristen was appointed as a Keck fellow by the Gulf Coast Consortia/Keck Center Pharmacoinformatics Training Program in 2008, and this fellowship was renewed for 2 years. Kristen's research on the IGFBP2 signaling pathway in glioma has led to the following publication: Holmes KM, Annala M, Chua YX, Dunlap SM, Liu X, Hugen N, Moore LM, Cogdell DE, Hu L, Nykter M, Hess K, Fuller GN, and Zhang W. IGFBP2-driven glioma progression is prevented by blocking a clinically significant network of integrin, ILK, and NF-κB. *Proc Natl Acad Sci U S A*. 2012 Feb 28;109(9):3475-80.