ROLES OF THE NF-κB PATHWAY IN GLIOBLASTOMA STEM CELLS AND CHORDOMA

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ABSTRACT

Amanda L. Rinkenbaugh: Roles of the NF-κB Pathway in Glioblastoma Stem Cells and Chordoma
(Under the direction of Albert S. Baldwin, Jr.)

The NF-κB pathway consists of a family of five transcription factors: RelA/p65, RelB, c-Rel, p100/p52, and p105/p50. Originally discovered for its involvement in inflammation and immune signaling, aberrant constitutive NF-κB activation is seen in many tumor types. NF-κB-dependent target gene regulation mediates several hallmarks of cancer, including survival, suppression of apoptosis, and invasion. This work examines NF-κB signaling in both glioblastoma and chordoma samples. In the first project, NF-κB is found to mediate cancer stem cell maintenance in glioblastoma explants. Both genetic and pharmacological NF-κB inhibition impair neurosphere formation at limiting dilutions. Use of an *ex vivo* brain slice co-culture model confirmed the *in vitro* findings, providing a novel platform for drug testing in glioblastoma studies that bridges the gap between cell culture and intracranial animal models. In the second project, NF-κB regulates proliferation and invasion of chordoma cell lines. Due to the rarity of chordomas, these tumors have not been well-characterized at a molecular level. These results provide some of the early evidence for NF-κB activation, potentially through regulation of IL-6, IL-8, and MMP9. Both of these studies suggest that IKK/NF-κB inhibition could provide therapeutic benefit in glioblastoma and chordoma, affecting multiple phenotypes that drive the poor prognosis of these tumors.

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LIST OF ABBREVIATIONS

AML: acute myeloid leukemia

APC: adenomatous polyposis coli

cIAP: cellular inhibitor of apoptosis

CML: chronic myeloid leukemia

CSC: cancer stem cells

DEN: diethylnitrosamine

DLBCL: diffuse large B cell lymphoma

ECM: extracellular matrix

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epithelial-mesenchymal transition

FGF: fibroblast growth factor

GBM: glioblastoma multiforme

GSC: glioblastoma stem cell

IκB: nuclear factor of kappa light polypeptide gene enhancer in B-Cells inhibitor

IKK: inhibitor of kappaB kinase

IL-6: interleukin-6

LPS: lipopolysaccharide

LZ: leucine zipper

MMP: matrix metalloproteinase

NBD: NEMO binding domain

NF-κB: nuclear factor of kappa light polypeptide gene enhancer in B-Cells

NIK: NF-κB-inducing kinase

PGE_2: prostaglandin E_2

PTEN: phosphatase and tensin homolog

POSTN: periostin

RANK: receptor activator of NF-κB

RHD: Rel homology domain

ROS: reactive oxygen species

STAT: signal transducer and activator of transcription

TAK1: TGF-β activated kinase 1

TAD: transcription activation domain

TLR: toll-like receptor

TNF: tumor necrosis factor

ZF: zinc finger

CHAPTER I

INTRODUCTION

1.1 Summary

The NF-κB transcription factor pathway is a crucial regulator of inflammation and immune response. Additionally, aberrant NF-κB signaling has been identified in many types of cancer. Downstream of key oncogenic pathways, such as RAS, BCR-ABL, and Her2, NF-κB regulates transcription of target genes that promote cell survival and proliferation, inhibit apoptosis, and mediate invasion and metastasis. The cancer stem cell model posits that a subset of tumor cells (cancer stem cells) drive tumor initiation, exhibit resistance to treatment, and promote recurrence and metastasis. This chapter examines the evidence for a role for NF-κB signaling in glioblastoma and chordoma, with a particular emphasis on cancer stem cell biology.

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1.2 NF-κB Signaling

The NF-κB family of transcription factors consists of five members: p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52. Each of these proteins contain a conserved N-terminal Rel homology domain which enables nuclear localization, DNA binding, and homo- and heterodimerization (Figure 1.1). p65, RelB, and c-Rel feature transcription activation domains as well, while p50 and p52 do not. The precursors p105 and p100 include ankyrin repeats which are proteolytically cleaved to produce the active subunits p50 and p52, respectively (Hayden and Ghosh, 2008). NF-κB signaling typically operates through two major pathways: the canonical and the non-canonical. Activation of the canonical pathway occurs downstream of many stimuli, including LPS or pro-inflammatory cytokines such as TNF or IL-1. Under basal conditions, p65-p50 dimers are sequestered in the cytoplasm by inhibitor of kappaB proteins (IκBs; Figure 1.2). Upon activation, the inhibitor of kappaB kinase (IKK) complex, which consists of the kinase subunits IKKα and IKKβ plus the regulatory subunit IKKγ (NEMO), phosphorylates IκBα, leading to its ubiquitination and proteasomal degradation (Figure 1.3). Loss of IκBα allows NF-κB nuclear localization, promoting transcription of its target genes, including anti-apoptotic factors, cytokines such as IL-6, and proliferation factors such as cyclin D1 (Figure 1.4). One group of target genes includes negative regulators of NF-κB signaling, such as A20 and IκBα. By producing these components, NF-κB generates a negative feedback loop to add another dimension of control to this pathway. The non-canonical pathway is activated through developmental signals such as BAFFR, CD40, or LTβR. Here, p100 acts like an IκB molecule, holding RelB in the cytoplasm. Non-canonical signaling leads to stabilization of NF-κB-inducing kinase (NIK). NIK activates IKKα dimers, which subsequently phosphorylate p100. p100 phosphorylation

leads to its cleavage into p52, producing an active RelB-p52 dimer that moves to the nucleus and regulates transcription (Figure 1.4) (Ghosh and Hayden, 2012).

1.3 NF-κB in Cancer

1.3.1 NF-κB activation in cancer

In addition to its roles in the innate immune system and inflammatory signaling, the NF-κB pathway has been extensively tied to cancer biology. The discovery of v-rel, the oncoprotein in an avian Rev-T virus responsible for reticuloendotheliosis, and its identification as the homolog of c-rel provided the first link between cancer and NF-κB (Kieran et al., 1990; Wilhelmsen et al., 1984). Early studies showed that NF-κB is activated downstream of oncogenic RAS and BCR-ABL, where it promotes the oncogenic phenotype (Finco et al., 1997; Reuther et al., 1998; Stein and Baldwin, 2011). Inhibition of NF-κB in oncogenic RAS+ cells leads to apoptosis, consistent with a role for NF-κB in driving an anti-apoptotic, pro-survival phenotype (Mayo et al., 1997). Many studies demonstrate that NF-κB and its target genes are upregulated in the majority of cancers – including both hematological malignancies and solid tumors. More recently, NF-κB has been shown to be activated downstream of loss of tumor suppressors such as p53, VHL, and PTEN (An and Rettig, 2005; An et al., 2004; Asano et al., 2004; Cooks et al., 2013; Di Minin et al., 2014; Gustin et al., 2001; Qi and Ohh, 2003; Weisz et al., 2007; Ying et al., 2011). While early efforts focused on analysis of canonical NF-κB signaling in cancer, recent studies indicate that non-canonical NF-κB signaling can also be found activated in different cancers (Lee et al., 2013; Li et al., 2015; Thu et al., 2011; Uno et al., 2014; Wang et al., 2007; Wharry et al., 2014; Xu

et al., 2009). Expression of the superrepressor form of IκBα (serines 32/36 mutated to alanines, preventing phosphorylation and degradation and leading to decreased NF-κB activity; IκBα-SR) and genetic deletion of IKKβ or RelA in RAS-driven lung tumor and melanoma models strongly suppressed tumor growth (Bassères et al., 2010; Meylan et al., 2009; Yang et al., 2010).

Once activated, NF-κB regulates a wide variety of target genes that overlap heavily with the hallmarks of cancer (Hanahan and Weinberg, 2011). Proliferation is one of the most basic characteristics of a cancer cell and NF-κB is involved through regulation of CyclinD1, Cyclin E, and c-Myc. NF-κB promotes survival and inhibits apoptosis through several mechanisms (Baldwin, 2012). These include transcriptional regulation of the cellular inhibitor of apoptosis (cIAPs) 1, 2, and XIAP, as well as Bcl-2 and Bcl-xL (Chu et al., 1997; Ramakrishnan et al., 2010; Wang et al., 1998). Perhaps as expected, NF-κB regulates a number of cytokines that contribute to tumor-promoting inflammation such as: TNFα, IL-1, IL6, MCP1, COX2, and iNOS. Other NF-κB targets contribute to epithelial-mesenchymal transition (vimentin, Twist), remodeling the extracellular matrix through induction of angiogenesis (IL8, VEGF), and promotion of invasion and metastasis (MMP2, MMP9, uPA) (Bassères and Baldwin, 2006).

The studies described above led to efforts to determine if human tumors feature genetic alterations in IKK/NF-κB components. Somewhat surprisingly, such mutations are not common. However, the level of coverage provided by next-generation sequencing has found examples of NF-κB-associated mutations in a low percentage of cancers, predominately hematological malignancies. Amplifications of c-rel, IKKβ, IKKγ, and the related kinase IKKε have been identified primarily in lymphomas and breast cancer (Beroukhim et al.,

2010; Boehm et al., 2007; Orlowski and Baldwin, 2002). Rearrangements of the NFKB2 locus (gene name for the p100 subunit) that lead to loss of the inhibitory IκB-like domain and increased p52 production are found in some B cell lymphomas (Neri et al., 1991). C11orf95-RELA fusions have been described as driver events in ependymomas (Parker et al., 2014), while monoallelic deletions of IκBα were identified in a subset of glioblastoma (Bredel et al., 2011). Mutations in upstream proteins that lead to aberrant, constitutive NF-κB activation have been identified. For example, in certain subtypes of lymphoma, translocations can affect MALT1 and BCL10, while CARD11 features a variety of point mutations. All three of these proteins interact to form a complex that drives NF-κB activation (Lim et al., 2012). Growth factor receptors, including EGFR and Her2, are frequently overexpressed in cancer and activate similar pathways, including NF-κB (Merkhofer et al., 2010; Tanaka et al., 2011).

IKK exhibits NF-κB-independent functions that promote growth and survival functions important to a variety of cancer cells. For example, IKKα and IKKβ promote mTOR activation, via kinase activity (Dan et al., 2007; 2008; 2014; Xu et al., 2013). Another example is that IKKα was found to phosphorylate the CDK inhibitor p27 downstream of Her2 to promote cancer stem cell self-renewal (Zhang et al., 2013). IKKβ was reported to phosphorylate the tumor suppressor p53 to promote its instability (Xia et al., 2009).

1.3.2 Chronic inflammation as a precursor to cancer

Another line of research linking NF-κB with oncogenesis examines the connection between chronic inflammation and tumor development. The microenvironment surrounding a tumor includes fibroblasts, infiltrating immune cells, extracellular matrix proteins and cytokines that interact with the tumor cells. In a mouse model of colitis-associated colon

cancer with targeted IKKβ deletion in either the epithelial or myeloid compartments, NF-κB mediated survival of intestinal epithelial cells, while NF-κB activation in myeloid cells drove production of growth factors that promoted tumor proliferation (Greten et al., 2004). In a model of hepatocellular carcinoma driven by treatment with the carcinogen diethylnitrosamine (DEN), NF-κB is again activated in the myeloid compartment, this time to drive IL6 production and subsequent STAT3 activation in the hepatocytes (Maeda et al., 2005). Interestingly, this model shows that deletion of IKKβ or IKKγ actually leads to enhanced tumor development. The liver initially shows more cell death following DEN treatment; however because hepatocytes are highly regenerative, the cell death triggers proliferation of the remaining cells (Sakurai et al., 2006). It is thought that a cycle of injury, cell death, and proliferation drives tumor formation in this model (DiDonato et al., 2012). NF-κB was shown to be activated in cancer-associated fibroblasts promoting the expression of inflammatory cytokines, although the role of this response in promoting tumorigenesis is controversial (Erez et al., 2010; Koliaraki et al., 2015; Pallangyo et al., 2015). Work examining tumor-associated macrophages has shown that NF-κB signaling maintains a tumor-promoting, immunosuppressive (or M2) phenotype and inhibits a tumor-suppressing (or M1) phenotype (Hagemann et al., 2008; Saccani et al., 2006). Taken together, these studies start to describe a complex microenvironment with multiple cell types interacting to drive tumorigenesis and place NF-κB as a central mediator between these various components.

1.4 Glioblastoma

Glioblastoma (GBM) is the most common primary adult brain tumor. Standard of

care treatment currently involves a combination of surgery, radiation, and chemotherapy, however median survival for these patients is only 14.6 months. Improvements in GBM treatment have not been frequent or substantial. In addition to the difficulties that accompany developing any new cancer therapy, GBM provides the extra hurdle of blood-brain barrier penetrance. While some drugs may show anti-tumorigenic activity *in vitro*, many are unable to cross the blood-brain barrier, and thus never reach the cancer cells they are supposed to target. The approval of temozolomide as a new chemotherapeutic treatment option in 2005 was one of the biggest additions in several decades of GBM research, yet the added survival benefit was a mere 2.5 months (Stupp et al., 2005).

Histopathologically, GBM is described as a heterogeneous tumor type with high levels of angiogenesis and invasion. The Cancer Genome Atlas (TCGA) analyzed around 200 GBMs and based on gene expression patterns clustered them into four subtypes: neural, proneural, classical, and mesenchymal (Verhaak et al., 2010). The most common genetic alterations associated with these tumors include EGFR amplification and mutation, loss of PTEN, and loss of p53, all of which have been associated with increased NF-κB activity (Brennan et al., 2013; Cooks et al., 2013; Gustin et al., 2001; Kaus et al., 2010; Tanaka et al., 2011; Verhaak et al., 2010). In GBM, NF-κB has been reported to regulate survival, invasion, and resistance to both radiation and chemotherapy (Atkinson et al., 2009; Bredel et al., 2006; Fukushima et al., 2012; Holmes et al., 2012; Li et al., 2010; Raychaudhuri et al., 2007; Tanaka et al., 2011; Xi et al., 2015).

1.5 Chordoma

Chordoma is a rare tumor type that arises from embryonic notochord remnants and has an incidence of less than one per million people (Smoll et al., 2013). As such, it has not

been well-studied and the molecular underpinnings of these tumors remain fairly uncharacterized when compared to other tumor types, such as glioblastoma. Though the tumors are relatively slow-growing, they present other clinical challenges as the anatomical location in the central nervous system makes surgery more difficult. Additionally, they are resistant to chemotherapy and radiation (Chugh et al., 2007; Forsyth et al., 1993). Furthermore, they tend to recur and are both locally invasive and capable of metastasis, particularly to the lungs, bone, and liver. Current treatment generally includes radical surgery and high-dose radiation; however treatment is not standardized due to low patient volume and lack of molecular characterization.

One of the major molecular determinants that has been associated with chordoma is brachyury expression. Brachyury (gene name *T*) is a T-box transcription factor expressed in the notochord during development, as well as specifically expressed in chordoma (Vujovic et al., 2006). Its expression is actually a requirement for the validation of pathology specimens or cell lines as bona fide chordomas. In familial chordoma, germline gene duplication of the *T* locus was identified in several families (Yang et al., 2009). Subsequently, a SNP within *T* was found to be present in 86% of chordoma patients versus 56% in controls (Pillay et al., 2012). Interestingly, this allele appears to affect both expression levels and DNA binding of the transcription factor, consistent with a functional role in chordoma development (Papapetrou et al., 1997).

Invasion poses a major challenge in treatment, as it can lead to destruction of adjacent normal tissue and makes it difficult to fully resect the primary tumor during surgery, especially given the proximity of crucial, delicate tissues near the base of the skull and along the spinal cord. One aspect of invasion is degradation of the extracellular matrix (ECM) to

allow the tumor cells to disseminate from the primary tumor. Many factors have been associated with this process including the matrix metalloproteinases (MMPs). MMPs are able to break down a wide variety of substrates, including collagen, gelatin, fibronectin, and laminin. Epidermal overexpression of type I collagenase (MMP1) in a carcinogenesis model led to a significant increase in tumor incidence (D'Armiento et al., 1995). Conversely, deletion of MMP7 in the Min/+ model of colon cancer led to a marked decrease in tumor formation (Wilson et al., 1997). Both of these results demonstrate involvement of the MMP family in promotion of cancer progression, but do not specifically involve invasive phenotypes. More recent studies have found that MMP9 expression is upregulated in invasive skin cancer lesions and MMP7 progressively accumulates as pancreatic tumors become metaplastic (Crawford et al., 2002; Kupferman et al., 2000). In chordoma, both MMP1 and MMP2 expression has been correlated with increased infiltration of bone as well as poor prognosis in patient specimens (Naka et al., 2004; 2008).

1.6 Cancer Stem Cells

Given the connections between the NF-κB pathway and the earliest events in oncogenesis, it follows that NF-κB signaling would be important in the tumor initiating cells. The cancer stem cell (CSC) model has been proposed to describe the cells which are responsible for tumor initiation. This phenomenon was first described in acute myeloid leukemia (AML), where cells from patients were transplanted into NOD/SCID mice and monitored for engraftment. Results from that study demonstrated that the $CD34^+$ $CD38^-$ population of cells caused disease more frequently and at lower cell numbers than $CD34^-$ cells (Bonnet and Dick, 1997). Subsequent to these findings, CSCs have been described in

many solid tumors including those of the brain, prostate, breast, colon, and pancreas (Al-Hajj et al., 2003; Collins et al., 2005; Li et al., 2007; Ricci-Vitiani et al., 2006; Singh et al., 2003; 2004). In addition to being responsible for primary tumor formation, CSCs are also generally thought to drive metastasis and exhibit increased resistance to radiation and chemotherapy. Due to their stem-like characteristics, these cells are also capable of differentiation into multiple lineages, which accounts for some of the heterogeneity seen in tumors (Reya et al., 2001). While CSCs are frequently depicted at the top of a hierarchically arranged tumor, there is evidence that plasticity allows for the conversion of bulk tumor cells into CSCs (Chen et al., 2010).

Several assays allow for the study of CSCs. *In vitro* experiments focus on sphere formation under stem cell permissive conditions, such as serum-free media supplemented with essential growth factors and low-adherence plates. Ideally, these experiments are performed at limiting dilutions to best assess self-renewal from single cells. Additionally, *in vivo* assessments of tumor formation remain the gold standard for true CSC function, again preferably performed under limiting dilutions (Bruce and van der Gaag, 1963; Hemmati et al., 2003; Nguyen et al., 2012; Reynolds and Weiss, 1992). Frequently, prospective CSCs can be isolated from the bulk of the tumor cells based on one or more markers, either through the use of magnetic beads or fluorescence-activated cell sorting. Many markers have been proposed to distinguish CSCs from other tumor cells. While no individual marker is perfect, a few of the most commonly used markers include CD133, CD44, and EpCAM (Al-Hajj et al., 2003; Collins et al., 2005; Dalerba et al., 2007; Li et al., 2007; Sales et al., 2007; Singh et al., 2003). Once isolated, the populations can be compared in a number of phenotypic assays

to dissect the differences between the cell types. Proliferation, survival, and gene expression analyses are commonly measured.

1.6.1 NF-κB activation in CSCs

One of the earliest examples of NF-κB involvement in CSCs came from primary AML samples, where the $CD34^+$ cells showed enhanced NF-κB DNA binding that was not seen in regular hematopoietic stem cells (Guzman et al., 2001). Since that initial report, elevated or constitutive NF-κB activity has been seen in many tumor types. Prostate CSCs were found to express higher levels of acetylated and total p65, as well as a decrease in IκBα expression when compared to parental tumors (Rajasekhar et al., 2011). In glioblastoma, CSCs exhibited increased nuclear localization of p65 as compared with cells cultured under monolayer conditions (Garner et al., 2013). Tumorsphere-forming cells showed increased phosphorylation of p65, again consistent with elevated NF-κB signaling in this population of cells. In that study, inhibition of NF-κB reduced self-renewal and blocked xenograft tumor growth using a limiting dilution approach (Song et al., 2012). In addition to direct evidence of preferential NF-κB activation in CSC subsets of tumors, several groups have taken an unbiased approach of profiling gene expression and defining CSC-associated signatures. This has revealed an inflammatory signature, which can frequently be tightly associated with NF-κB regulation, in a variety of tumors such as glioblastoma, breast, prostate, and ovarian cancers (Birnie et al., 2008; Korkaya et al., 2011; Leizer et al., 2010; Liu et al., 2007; Murohashi et al., 2010; Tafani et al., 2011).

Perhaps not surprisingly, some of the same oncogenes previously mentioned to activate NF-κB also participate in the CSC subpopulations of tumors. In mouse models of Her2-

driven breast cancer, both canonical and non-canonical NF-κB pathways contribute to stemness and tumor formation. Expression of IκBα-SR impaired the formation of luminal epithelial tumors. Use of an NF-κB-GFP reporter allele localized activation to the luminal progenitors (Pratt et al., 2009). Another analysis of IκBα-SR in a Her2 mouse model found changes in a gene signature associated with stem cells, then specifically showed NF-κB-dependent changes in the specific stem cell factors Nanog and Sox2 (Figure 1.5) (Liu et al., 2010). Knock-in of a kinase dead IKKα led to decreased self-renewal and senescence under mammary stem cell culture conditions (Cao et al., 2007). In the Her2 breast cancer model, IKKα was found to phosphorylate p27 leading to its nuclear export and promoting CSC proliferation and expansion (Zhang et al., 2013). One of the canonical alterations that occurs during colorectal tumorigenesis is loss of APC. Myant and colleagues found that APC loss drives RAC1 activity to mediate ROS production and NF-κB activation, ultimately leading to an expansion of $Lgr5^+$ CSCs (Myant et al., 2013).

1.6.2 Connections between NF-κB signaling, cytokines, and CSCs

Signaling from toll-like receptors (TLRs) is known to drive traditional NF-κB activation in an inflammatory setting. In ovarian CSCs, TLR2-MyD88-driven NF-κB activity regulates expression of the stem cell associated genes CD44, Sox2 and Nanog (Chefetz et al., 2014). TLR9 drives the propagation and self-renewal of androgen-independent prostate CSCs, largely through the co-activation of the NF-κB and STAT3 pathways, which in turn regulate expression of the crucial stem cell transcription factors NKX3.1 and KLF4 (Moreira et al., 2015). Many cytokines have also been associated with supporting CSC maintenance in an NF-κB-dependent manner. Chronic myeloid leukemia (CML) stem cells produce higher

levels of TNFα than normal hematopoietic stem cells. Canonical NF-κB activation positively regulates expression of IL3 and granulocyte/macrophage colony-stimulating factor common β-chain receptor (CSF2RB) to promote proliferation and survival of CML stem cells (Gallipoli et al., 2013). Similar findings in a mouse model of acute myeloid leukemia (AML) described a feedback loop between TNFα and NF-κB, confirmed by correlations in patient samples (Kagoya et al., 2014). TNFα treatment of MCF7 breast cancer cells increased their mammosphere-forming capacity through upregulation of NF-κB and subsequently Slug (Figure 1.5) (Storci et al., 2010). In colorectal cancer, levels of prostaglandin E_2 (PGE_2) correlated with CSC markers in human tumor samples. Treatment of either a genetic or xenograft mouse model with PGE_2 led to CSC expansion through upregulation of several signaling pathways including NF-κB (Wang et al., 2015). In glioblastoma, IL-17 receptor was found to be co-expressed with multiple CSC markers, including CD133, Nestin, and Sox2, as well as a source of NF-κB activation (Parajuli et al., 2016).

While several cytokines drive NF-κB signaling, NF-κB also controls the expression of a variety of other cytokines, particularly IL-6 and IL-8, that are heavily associated with CSC function. Iliopoulous and colleagues studied an inducible model of transformation by Src in mammary epithelial cells that led to rapid secretion of IL6 and increased NF-κB activation. NF-κB positively regulates Lin28 transcription, which in turn decreases the level of let-7 microRNA. As IL6 is one target of this microRNA, IL6 expression increases even further, creating a positive feedback loop driving transformation and CSC expansion (Iliopoulos et al., 2009; 2011). Interestingly, let-7 also targets KRas, and decreased levels of let-7 have been shown to drive mammosphere formation and size through Ras-NF-κB and Ras-MAPK-ERK pathways (Xu et al., 2015). In basal-like breast cancer, NF-κB inhibition decreases

mammosphere formation, but addition of exogenous IL6 or IL-1β rescues the defect (Kendellen et al., 2013). In CML, increased levels of IL6 drive CML progenitors into the myeloid lineage, sustaining CML development (Reynaud et al., 2011). IL6, IL8, and MCP1 similarly contribute to the survival and self-renewal of glioblastoma CSCs (Figure 1.5) (Parajuli et al., 2016; Wang et al., 2009).

1.6.3 Interactions between NF-κB and the tumor microenvironment

Given the close association between NF-κB and cytokines, it reasonably follows that NF-κB plays a role in modulating the microenvironment. CSCs are thought to occupy certain niches within tumors, much like their normal stem cell counterparts. For example in glioblastoma, CSCs have been localized to a perivascular niche, populated by an abundance of proliferating stromal and endothelial cells that specifically support the growth of CSCs (Calabrese et al., 2007; Charles et al., 2010). As previously mentioned, preferential expression of IL-17 receptor is seen on glioblastoma CSCs. Relatedly, in ovarian cancer, macrophages and CD4+ T cells produce IL-17 to drive self-renewal of CSCs *in vitro* and tumor formation *in vivo* in an NF-κB- and p38-dependent manner (Xiang et al., 2013). Interestingly, there is evidence for CSCs promoting angiogenesis through secretion of endothelial factors like VEGF and IL8 or through direct transdifferentiation (Alvero et al., 2009; Ricci-Vitiani et al., 2006; Soda et al., 2011; Wang et al., 2010). Osteopontin is an oncoprotein that signals through integrins as well as CD44 family receptors, which have been used as a CSC marker in several tumor types. Hepatocellular carcinoma stem cells exhibit enhanced expression of osteopontin which drives a transcriptional cascade from NF-κB activation to HIF1α to BMI1 expression (Cao et al., 2015). Periostin (POSTN) is a

component of the extracellular matrix that has been identified in the niche of both normal and cancer stem cells. Generally thought to be produced by stromal fibroblasts, POSTN promotes metastasis to the lung in a breast cancer model by supporting the growth and expansion of CSCs (Malanchi et al., 2012). Another group found that breast CSCs express higher levels of POSTN *in vitro* than their non-CSC counterparts. POSTN drives an ERK-NF-κB signaling axis, driving production of IL6 and IL8, which in turn contribute to CSC maintenance through STAT3 activation (Lambert et al., 2016). Breast cancer also exhibits a circuit of progestin-driven RANKL (receptor activator of NF-κB ligand) expression, leading to NF-κB activation. Deletion of the RANKL receptor RANK decreases the $CD49f^{hi}$-CSC population and tumor incidence (Figure 1.5) (Schramek et al., 2010).

1.6.4 Contributions by the NF-κB pathway to invasion and metastasis

In addition to creating the proper niche for CSC survival and expansion, NF-κB also contributes to the invasive and metastatic capabilities of CSCs. This can occur through further modulation of the extracellular environment or through cell-intrinsic changes like epithelial-mesenchymal transition (EMT) which has been linked to CSC characteristics (Mani et al., 2008). Work by several groups has shown NF-κB-mediated regulation of critical EMT factors including Snail(Barberà et al., 2004; Kim et al., 2007), Slug (Belguise et al., 2007; Kumar et al., 2013), ZEB1/2 (Chua et al., 2006), and Twist1 (Kanegae et al., 1998; Pham et al., 2007; Šošić et al., 2003; Takeda et al., 1999; Wang et al., 1997) (reviewed in (Min et al., 2008)). TNFα leads to NF-κB-dependent stabilization of Snail and transcriptional upregulation of Twist1, both of which enhanced invasion *in vitro* and metastasis *in vivo* (Li et al., 2012; Wu et al., 2009). Inhibition of NF-κB led to a reversal of EMT in mammary

epithelial cells and decreased metastasis in an *in vivo* model (Huber et al., 2004). In breast cancer, overexpression of RANK drives EMT and expansion of the $CD44^{+}/CD24^{-}$ CSC population, ultimately leading to increased tumor growth and a substantially higher number of metastases (Palafox et al., 2012). Another study found overexpression of AXL in breast cancer stem cells; inhibition of AXL decreased NF-κB activity, expression of EMT-associated genes, invasion, and tumor formation (Asiedu et al., 2013). In non-small cell lung cancer (NSCLC), Kumar and colleagues induced EMT through dual treatment with TNFα and TGF-β. The associated EMT transcription factors Twist1, Slug, and ZEB2 were upregulated in an NF-κB-dependent manner, followed by increases in multiple stem cell factors: KLF4, SOX2, POU5F1, MYCN, and KIT (Kumar et al., 2013). Subsequent studies found that NF-κB-mediated upregulation of Activin was required in order to maintain the mesenchymal phenotype of NSCLC CSCs (Wamsley et al., 2015). There is also evidence that signaling through the NF-κB pathway and CXCR4 maintains stemness and promotes migration (Es-haghi et al., 2015; Helbig et al., 2003; Singh et al., 2012; Zhi et al., 2014). NF-κB has also been found to regulate the expression of matrix metalloproteinases (MMPs), which can degrade components of the extracellular matrix to increase invasion of tumor cells. Specifically, NF-κB directly regulates transcription of MMP9 (Farina et al., 1999; Himelstein et al., 1997; Ricca et al., 2000), while indirectly increasing MMP2 activity (Connelly et al., 2007; Han et al., 2001; Philip et al., 2001). Ovarian CSCs upregulate MMP9 expression to enable invasion and metastasis downstream of CCL5-NF-κB signaling (Long et al., 2012). NF-κB has also been shown to regulate VEGF and IL-8, which promote tumor formation and angiogenesis (Huang et al., 2000).

NF-κB frequently cooperates with additional signaling pathways to mediate these oncogenic effects. Coordinated activity between NF-κB and STAT3 has been previously mentioned in this review. Concurrent constitutive signaling from NF-κB and STAT3 in glioblastoma CSCs regulates expression of a set of genes (NOTCH1, HES5, JAG1, NUMBL, DTX3, DVL3, and RBPJ) that drive activation of Notch signaling, a third CSC-associated pathway (Figure 2) (Garner et al., 2013). Another experiment, suggesting an important interaction between the CSCs and the bulk tumor cells, found that NF-κB activity in the non-CSCs upregulates JAG1 to stimulate Notch signaling in proximal breast CSCs (Yamamoto et al., 2013).

The majority of the findings discussed here have focused on the canonical NF-κB pathway, particularly the p65 subunit. However, there is also evidence that the non-canonical pathway contributes to CSC phenotypes. In breast cancer, knockdown of IKKα, p100/p52, or RelB all produced a decrease in mammosphere formation (Kendellen et al., 2013). Eva1, found to be overexpressed in glioblastoma CSCs, drives NIK stabilization and p100 processing, potentially by promoting ubiquitination and degradation of TRAF2 and cIAP (Ohtsu et al., 2016). RelB has been described as an oncogenic driver in mesenchymal glioma, regulating Olig2 expression and promoting tumor growth and invasion (Lee et al., 2013).

1.7 NF-κB as a Therapeutic Target

Given the extensive ties between NF-κB signaling and CSC biology, there has naturally been an interest in targeting the pathway therapeutically. In several of the studies previously mentioned, either knockdown of pathway components or targeted inhibitors produced a decrease in stem cell phenotypes *in vitro* as well as decreased tumor growth

and/or metastasis *in vivo*. A combination of idarubicin and MG132, a proteasome inhibitor, induced cell death in AML stem cells, partially through NF-κB inhibition (Guzman et al., 2002). While proteasome inhibition will impact several pathways in a cell, NF-κB inhibition is a well-established effect of MG132 treatment as it blocks IκBα degradation and effectively sequesters NF-κB subunits in the cytoplasm. The same group went on to identify the compound parthenolide as selectively inducing apoptosis in leukemia stem cells as opposed to normal hematopoietic stem cells, through a mechanism of increased reactive oxygen species, p53 activation, and NF-κB inhibition (Guzman et al., 2005). A subsequent *in silico* screen for additional drugs with specificity towards AML stem cells identified two other compounds, celastrol and 4-hydroxy-2-nonenal, and once again they found NF-κB inhibition to be part of the mechanism of action (Hassane et al., 2008). Parthenolide has also shown preferential activity in breast CSCs compared to the bulk tumor cells (Zhou et al., 2007). Use of SN50, a peptide inhibitor that blocks nuclear import of NF-κB and other transcription factors, decreases the sphere formation ability and tumorigenic capacity of glioma CSCs (Zhang et al., 2014). Others have found that inhibition of NF-κB promotes more rapid differentiation and progression to senescence in glioblastoma CSCs (Nogueira et al., 2011).

The activated B-cell subtype of diffuse large B-cell lymphoma (DLBCL) has shown a distinct dependence on NF-κB signaling. Standard treatment for lymphoma patients includes rituximab, a monoclonal antibody against CD20. While this drug has many effects, one aspect includes inhibition of NF-κB signaling to induce apoptosis. More recently, ibrutinib, a BTK inhibitor, has been found to improve patient outcome in clinical trials. While this drug does not specifically target IKK, BTK represents a key intermediate between B cell receptor and NF-κB, and ibrutinib treatment decreases NF-κB signaling (Akinleye et al., 2013). Other

studies have found an impact of IKK/NF-κB inhibition on tumor growth. While this work didn't specifically analyze CSC effects, if CSCs are primarily driving tumor initiation, we could interpret these results as having some effect on the CSC population. Direct IKKβ inhibitors showed efficacy in a mutant KRas, p53-null model of lung cancer (Bassères et al., 2014; Xue et al., 2011). In addition to inhibitors targeting the kinase activity, the NF-κB pathway can be inhibited by peptides encompassing the NEMO-binding domain (NBD) that block association of the IKK catalytic subunits with NEMO/IKKγ. Recently, use of an NBD peptide slowed tumor growth in both a human glioma xenograft and a genetic mouse model of glioma (Friedmann-Morvinski et al., 2016). The NBD peptide has also shown efficacy in a canine model of DLBCL (Gaurnier-Hausser et al., 2011; Habineza Ndikuyeze et al., 2014).

NF-κB signaling also has ties to mediating resistance to radiation and chemotherapy, so there could be utility in combining NF-κB inhibition with traditional cancer therapies. Early work found that expression of IκBα-SR sensitized cancer cells to ionizing radiation, daunorubicin, and CPT-11 (a topoisomerase inhibitor) (Cusack et al., 2001; Wang et al., 1999; 1996). More recently, use of NF-κB inhibitors in combination with temozolomide, adriamycin, or radiation has shown increased apoptosis in glioblastoma cells (Brassesco et al., 2013; Fukushima et al., 2012; Shukla et al., 2013). Doxorubicin-resistant glioblastoma stem cells upregulated expression of MDR1 through a PI3K-NF-κB pathway (Xi et al., 2015). In another study, KRas-NF-κB signaling mediated resistance to EGFR inhibitors in CSCs (Seguin et al., 2014). Upregulation of IRAK1 drives NF-κB activation and cytokine production, leading to CSC enrichment and paclitaxel resistance in breast cancer (Wee et al., 2015). Taken together, these results suggest that not only could NF-κB inhibition be an

effective treatment against CSCs, but it could also restore sensitivity to other therapeutic options.

1.8 Conclusions

The NF-κB pathway is integrated into many critical aspects of tumor biology. Its function in inflammation and immune response often seems to set the stage for tumor development. Expression of several potent oncogenes, including mutant RAS and BCR-ABL, leads to NF-κB activation early in tumorigenesis. Here, we have detailed crucial roles and contributions of NF-κB in cancer stem cells, which drive tumor initiation, recurrence, invasion, and metastasis. NF-κB regulation of critical target genes, prominently including IAPs, MMPS, cytokines, and EMT transcription factors, drive CSC phenotypes. In addition to direct NF-κB effects, there is also cooperation between other crucial CSC-associated pathways, such as STAT3, Notch, and TGF-β. Future work will need to determine if therapeutic targeting of the NF-κB pathway impacts tumor growth in glioblastoma and chordoma, particularly at the level of cancer stem cells.

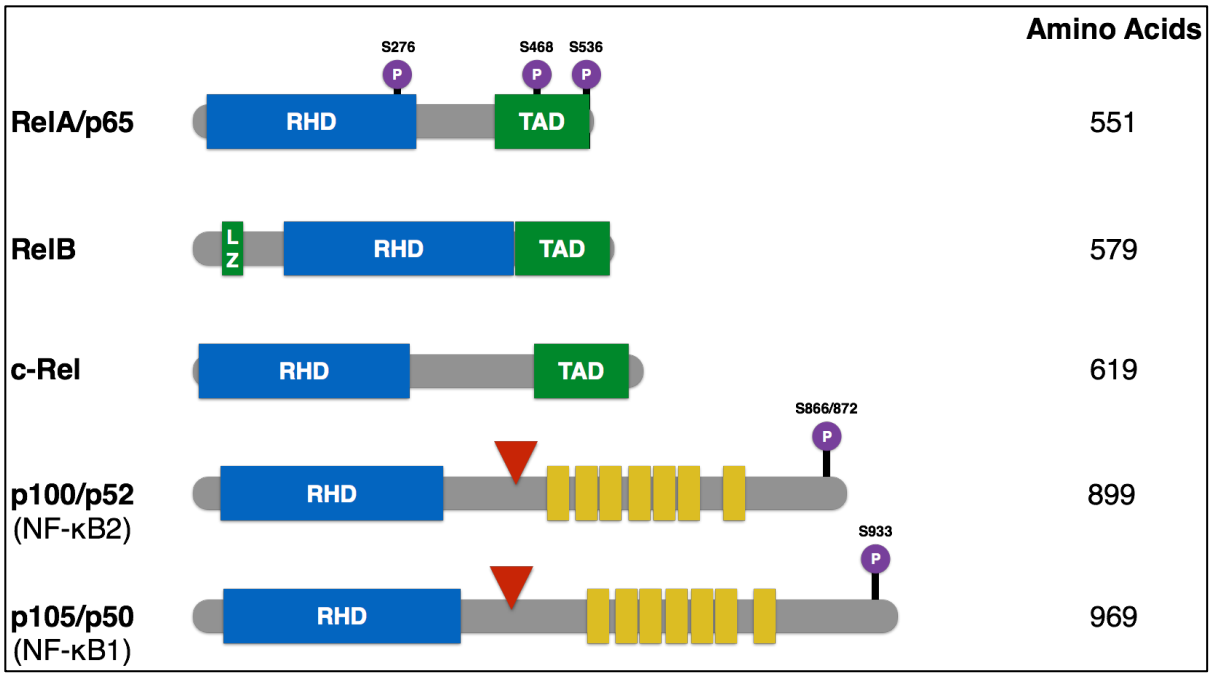

Figure 1.1 Domain organization of NF-κB transcription factor family members

The five members of the NF-κB family of transcription factors are RelA/p65, RelB, cRel, p100-p52, p105-p50. The defining feature of this family is the Rel homology domain (RHD, blue), which mediates homo- and hetero-dimerization of the subunits, nuclear localization, and DNA binding. RelA, RelB, and c-Rel also all contain a transcription activation domain (TAD, green). p100 and p105 are precursor proteins that contain several ankyrin repeats (yellow boxes), which act in an IκB-like manner to keep NF-κB in the cytoplasm. Upon phosphorylation (purple circles), these proteins are proteolytically degraded to release the active subunits p52 and p50, respectively (red triangles).

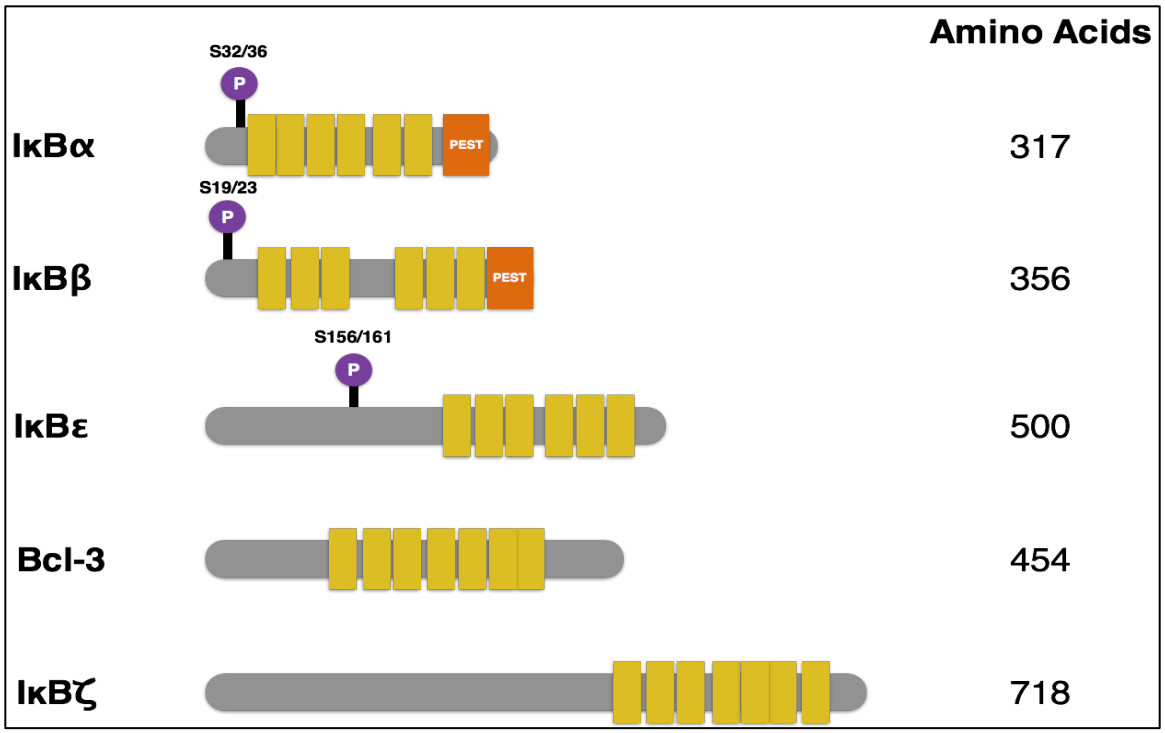

Figure 1.2 Domain organization of IκB family members

The IκB molecules are primary inhibitors of the NF-κB transcription factors. They are characterized predominately by several ankyrin repeats (yellow boxes) which mask the nuclear localization signal (NLS) in its binding partners. The typical IκBs are IκBα, β, and ε, which sequester NF-κB dimers in the cytoplasm. Once phosphorylated (purple circles), these proteins are ubiquitinated and proteasomally degraded to release NF-κB, which is partially mediated through the C-terminal PEST domain. There are also atypical IκBs, such as Bcl-3 and IκBζ, which share homology through the ankyrin repeats, but do not fill similar roles in the cell.

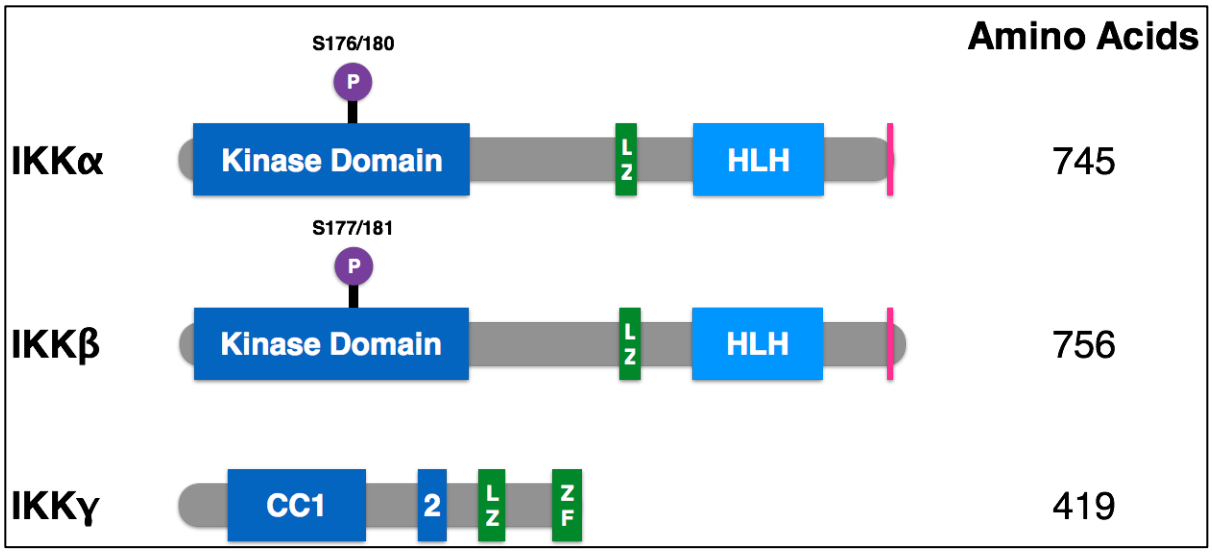

Figure 1.3 Domain organization of IKK family members

The canonical IKK complex consists of three proteins: IKKα, β, and γ. As seen here, IKKα and β are kinases within the helix-loop-helix (HLH) group. They also contain a NEMO binding domain (NBD; pink rectangle) which mediates binding to IKKγ/NEMO, and a leucine zipper (LZ) domain. The phosphorylation sites of S176/180 or S177/181 are in the activation loop of these kinases and are required for kinase activity. IKKγ contains two coiled coil domains (CC1 and CC2), a leucine zipper, and a zinc finger (ZF).

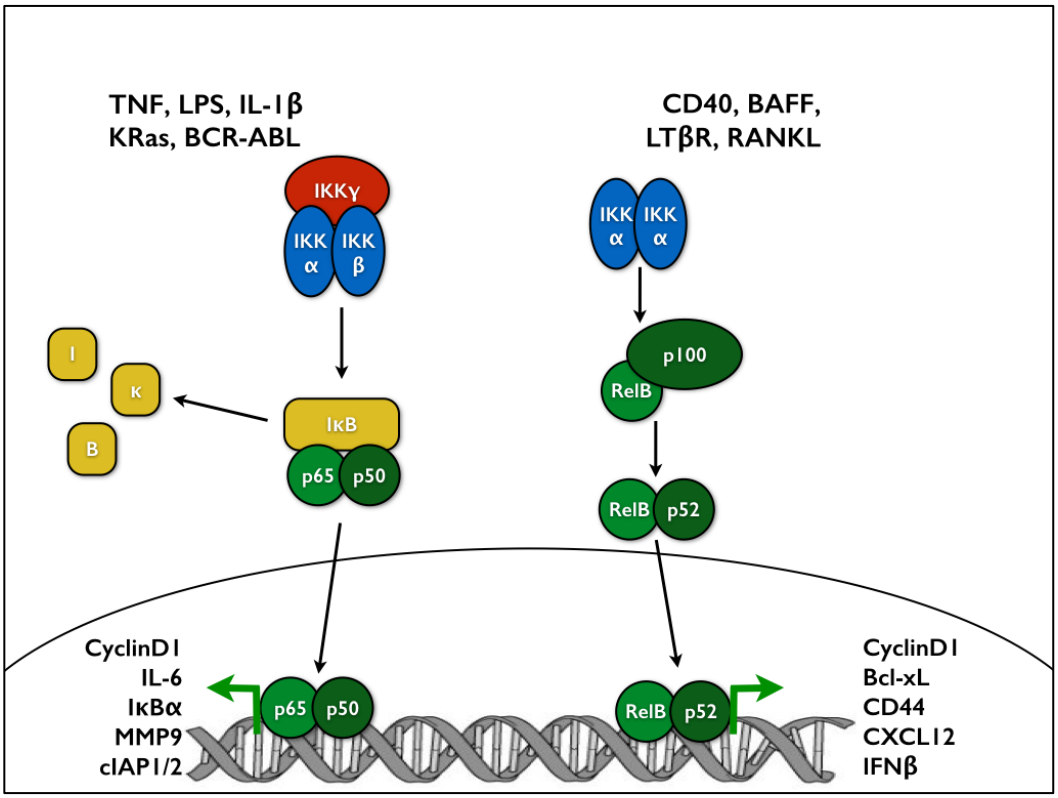

Figure 1.4 Canonical and non-canonical NF-κB signaling pathways

NF-κB signaling consists of two main branches: canonical and non-canonical. On the left, canonical NF-κB is driven by the IKK complex containing IKKα, β, and γ subunits. Phosphorylation of IκBα leads to its degradation, leaving the p65-p50 dimer free to translocate to the nucleus and regulate transcription of target genes. On the right, non-canonical NF-κB is driven by IKKα homodimers, leading to p100 processing. Here the dimer consists of the RelB and p52 subunits. Canonical and non-canonical NF-κB subunits regulate expression of distinct, but overlapping sets of target genes (Bradford and Baldwin, 2014).

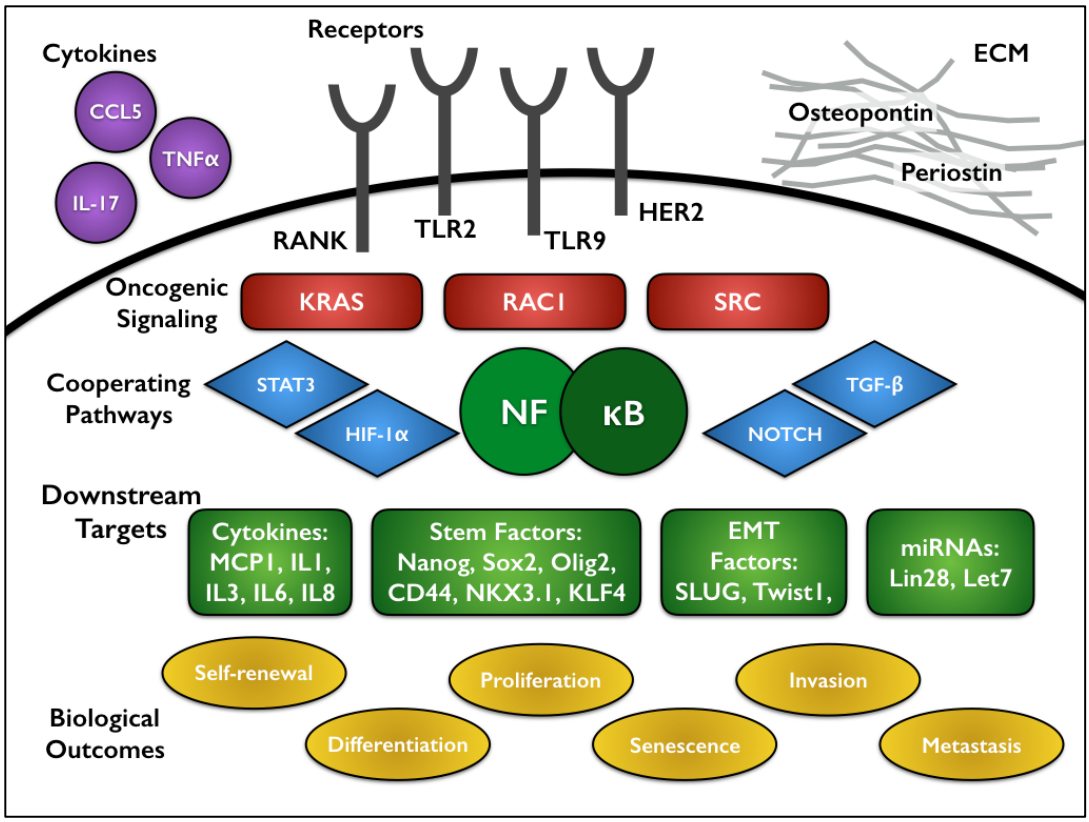

Figure 1.5 Overview of NF-κB pathway involvement in cancer stem cell biology

This diagram summarizes the various levels of NF-κB signaling in cancer stem cells. Both extracellular and intracellular sources of NF-κB activation are seen at the top. Either alone or in cooperation with other signaling pathways, NF-κB mediates a wide variety of transcriptional targets, which fall into several major categories such as cytokines and epithelial-mesenchymal transition factors. Ultimately, these targets mediate important aspects of cancer stem cell biology, including self-renewal, proliferation, and metastasis.

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CHAPTER II

IKK/NF-κB SIGNALING CONTRIBUTES TO GLIOBLASTOMA STEM CELL MAINTENANCE

2.1 Summary

Glioblastoma multiforme (GBM) carries a poor prognosis and continues to lack effective treatments. Glioblastoma stem cells (GSCs) drive tumor formation, invasion, and drug resistance and, as such, are the focus of studies to identify new therapies for disease control. Here, we identify the involvement of IKK and NF-κB signaling in the maintenance of GSCs. Inhibition of this pathway impairs self-renewal as analyzed in tumorsphere formation and GBM expansion as analyzed in brain slice culture. Interestingly, both the canonical and non-canonical branches of the NF-κB pathway are shown to contribute to this phenotype. One

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source of NF-κB activation in GBM involves the TGF-β/TAK1 signaling axis. Together, our results demonstrate a role for the NF-κB pathway in GSCs and provide a mechanistic basis for its potential as a therapeutic target in glioblastoma.

2.2 Introduction

Glioblastoma multiforme (GBM) is the most common primary brain tumor in adults, but despite multimodal treatment combining surgery, radiation, and chemotherapy, the median survival for patients remains under 15 months (Johnson and O'Neill, 2011). Pathologically, GBM is described as a heterogeneous tumor type with high levels of angiogenesis and invasion (Huse and Holland, 2010). Recent studies provide evidence for a population of cancer stem cells (CSCs) within GBM contributing to this heterogeneity (Singh et al., 2003; 2004). These cells (hereafter called GSCs) carry neural stem cells markers such CD133, self-renew over serial passages, and differentiate into multiple lineages. As with tumor-initiating cells described in other cancers (Hermann et al., 2007; Kumar et al., 2013; Long et al., 2012; Mani et al., 2008; Mueller et al., 2009; Todaro et al., 2007), GSCs been shown to be invasive and resistant to both radiation and chemotherapy (Bao et al., 2006; Cheng et al., 2011; Garcia et al., 2010; Liu et al., 2006). As such, there is significant interest in understanding the biology of the GBM GSC population to identify potential novel therapeutic targets to improve disease control.

NF-κB is a family of transcription factors consisting of five members: p65 (RelA), RelB, c-Rel, p105/p50, and p100/p52 that homo- and heterodimerize to regulate transcription of target genes. In the canonical pathway under basal conditions, p65-p50 dimers are bound to IκBα in the cytoplasm. Stimuli such as TNF-α or IL-1 lead to activation of the IKK complex, which consists of two catalytic subunits, IKKα and IKKβ, as well as a scaffolding subunit, IKKγ or NEMO. IKK phosphorylates IκBα, leading to its ubiquitination and proteasomal degradation, which allows NF-κB to accumulate in the nucleus. The non-canonical pathway is activated by developmental stimuli such as BAFF or CD40. Here, the

precursor p100 acts as an IκB molecule bound to RelB. Upon activation, IKKα phosphorylates p100, leading to its cleavage to produce p52. The active RelB-p52 dimer can then regulate transcription of target genes (Bradford and Baldwin, 2014; Hayden and Ghosh, 2008). Originally identified for its role in inflammatory signaling, the NF-κB pathway has since been demonstrated to be activated in various forms of cancer and is thought to contribute to the malignant phenotype through dysregulation of important biological processes such as proliferation, angiogenesis, apoptosis, and cell survival (Bradford and Baldwin, 2014; DiDonato et al., 2012; Lim et al., 2012; Perkins, 2012).

Within the nervous system, NF-κB is typically considered to be inactive, but activated in cases of injury or inflammation, consistent with its canonical function in other tissues (Kaltschmidt et al., 2005; Widera et al., 2008). In normal neural stem cells (NSCs), TNFα has been shown to induce proliferation through NF-κB (Widera et al., 2006). Kaus et al. have shown that as NSCs acquire the ability to proliferate independent of exogenous growth factors, these cells demonstrate increased NF-κB activity (Kaus et al., 2010). In GBM, NF-κB has been reported to regulate survival, invasion, and resistance to both radiation and chemotherapy (Bhat et al., 2013; Kesanakurti et al., 2012; Raychaudhuri et al., 2007; Tanaka et al., 2011; Thaker et al., 2009). *PTEN* deletion and *EGFR* amplification and/or mutation are two of the most common genetic alterations in GBM and both can lead to increased NF-κB activation (Tanaka et al., 2011; Verhaak et al., 2010). Additionally, TGF-β signaling has been demonstrated to contribute to GSC maintenance through the upregulation of LIF, Sox2, and Sox4 (Ikushima et al., 2009; Peñuelas et al., 2009). While the TGF-β and NF-κB pathways are thought to antagonize each other in some settings, there is evidence of their cooperation within tumors, including GBM (Song et al., 2012; Wang et al., 2015). Others

have identified alterations in the NF-κB pathway itself, with a subset of GBMs harboring monoallelic *NFKBIA* (gene name for IκBα) deletions and others expressing high levels of miR-30e* which targets IκBα (Bredel et al., 2011; Jiang et al., 2012). Consistent with the involvement of NF-κB signaling in GBM, recent work demonstrated that treatment with a NEMO-binding domain (NBD) peptide that blocks interactions between NEMO and IKKα/β slowed tumor growth in both a human glioma xenograft and a genetic glioma mouse model (Friedmann-Morvinski et al., 2016).

NF-κB activity has been associated with CSCs in several cancers (Guzman et al., 2001; Kendellen et al., 2013; Leizer et al., 2010; Murohashi et al., 2010; Pratt et al., 2009; Rajasekhar et al., 2011), and for GBM the NF-κB targets IL-6 and A20 have been shown to contribute to the maintenance GSCs (Hjelmeland et al., 2010; Wang et al., 2009). When cells are grown in CSC-permissive conditions instead of monolayers, there is an upregulation of NF-κB activity as seen through p65 phosphorylation and target gene expression (Garner et al., 2013). Other data suggest that inducing differentiation of GBM CSCs increases NF-κB activity. However, NF-κB inhibition accelerates differentiation, suggesting a role for this pathway in maintaining the cells in a more stem-like state (Nogueira et al., 2011).

In this study, we sought to investigate the role of the NF-κB pathway in GBM CSCs directly. We find that phosphorylation of the p65 (RelA) subunit of NF-κB is elevated in CD133+ GBM cells as compared to CD133-. Targeting NF-κB signaling either genetically or pharmacologically impairs self-renewal in primary tumorsphere assays and in limiting dilution assays. Interestingly, both canonical and non-canonical NF-κB pathways contribute to the GSC phenotype. Our results indicate that one source of NF-κB activation in GSCs is a TGF-β signaling pathway acting through TAK1. Using an *ex vivo* brain slice co-culture

model, we show that NF-κB contributes to the growth and survival of tumorspheres. Our findings indicate that NF-κB signaling is a key therapeutic target controlling GSCs.

2.3 Materials and Methods

Cell Culture, CD133+ Isolation, and Reagents

Human glioblastoma explants (6969, 7030, 7063, and GBM6) were obtained from Dr. William Weiss at UCSF and maintained in Neurobasal medium (Invitrogen, Carlsbad, CA), supplemented with B27 without Vitamin A, L-glutamine, 20ng/mL EGF, 40ng/mL FGF, and penicillin/streptomycin. To dissociate tumorspheres, cells were incubated with Accutase (Sigma, St. Louis, MO) in a 37°C water bath for 10 minutes, then plated as desired. To isolate CD133+ cells, cells were dissociated with Accutase and passed over a pre-separation filter to achieve single cell suspension. Dead cells were removed using the Dead Cell Removal Kit according to manufacturer's instructions (Miltenyi, Bergisch-Gladbach, Germany). Remaining cells were incubated with CD133 magnetic microbeads (Miltenyi, Bergisch-Gladbach, Germany) for 30 minutes, resuspended in MACS buffer and passed over two LS columns consecutively. Non-retained cells were saved for the CD133- fraction. After washing, the column was removed from the magnet and retained cells were expelled from the column, counted, and plated for experiments. Normal human astrocytes were a kind gift from Dr. Russell Pieper (Sonoda et al., 2001a; 2001b) and were maintained in DMEM media with 10% FBS and 1% penicillin/streptomycin. Neural stem cells (Millipore, Billerica, MA) were maintained in RenCell NSC maintenance media and grown on laminin-coated plates.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cellular proliferation assay.

Cells were seeded at 2000 or 3000 cells per well in 96-well plates, then treated with DMSO or Compound A daily as indicated. At each time point, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) compound (Promega, Madison, WI) was added and absorbance was read at 490 nm on a Versamax Microplate Reader (Molecular Devices, Sunnyvale, CA).

Luciferase Assay

Cells were transfected with 3x-κB luciferase reporter plasmid (Baldwin et al., 1991) using FuGENE HD (Promega, Madison, WI). Six hours post-transfection, cells were separated into 12-well plates and treated in duplicate with inhibitors as indicated for 24 hours. Cells were lysed in 150 μL of Passive Lysis Buffer, then 20 μL of lysate was used for analysis in triplicate using the Luciferase Assay System (Promega, Madison, WI). Luciferase signal was read on a Synergy2 plate reader (Biotek, Winooski, VT), and then normalized to protein content of each well based on a Bradford assay.

Western Blotting

Whole cell extracts were prepared by collecting cells, washing with cold PBS, then suspending in cold lysis buffer (1% NP-40, 20 mM Tris-Cl, pH 7.6, 138 mM NaCl, 2mM EDTA, 10% glycerol) on ice for 10 minutes, followed by 10 minutes of centrifugation to remove insoluble components. Protein was quantitated by Bradford assay (Biorad, Hercules, CA). Equal amounts of lysate (25-50 μg) were separated by SDS-PAGE, transferred to

nitrocellulose membranes, and blocked for 1 hour in 5% milk. Membranes were incubated with primary antibody overnight at 4°C, then incubated with secondary antibody for 1 hour at room temperature and developed using ECL reagent (GE, Fairfield, CT). Antibodies used were phospho-Smad2/3 (S465, 467/S423, 425), phospho-p65 (S536), p65, (Cell Signaling Technology, Danvers, MA) and β-tubulin (Santa Cruz Biotechnology, Dallas, TX).

siRNA Transfection

Human siRNA targeting *RELA* (M-003533-02), *RELB* (M-004767-02), *NFKB2* (M-003918-02), *IKBKB* (M-003503-03), *MAP3K7* (M-003790-06), or control #3 (D001201-03) was purchased from Thermo/Dharmafect (Lafayette, CO). DharmaFECT reagent 1 was used to transfect siRNA into cells according to the manufacturer's instructions. Six hours post-transfection, the media was changed on the cells. Cells were harvested 48-72 hours post-transfection for analysis by quantitative real-time PCR or Western blot.

Quantitative real-time PCR

RNA extracts were obtained from cells using the RNeasy Plus Kit (Qiagen, Hilden, Germany). Two micrograms of RNA were reverse transcribed using random primers and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed using Taqman Gene Expression Assay primer-probe sets for *GUSB* (Hs99999908_m1), *RELA* (Hs00153294_m1), *RELB* (Hs00232399_m1), and *NFKB2* (Hs01028901_g1) and relative quantification was determined using the $\Delta\Delta C_t$ method.

Tumorsphere and Limiting Dilution Assays

For tumorsphere assays, cells were dissociated into single cell suspension using Accutase. Cells were counted and plated at 100 cells per well in 24-well tissue culture plates. Cells were treated with inhibitors as indicated (once or daily for the duration of the experiment): 5 μM Compound A, 2.5 μM (5Z)-7-oxozeanol (Tocris, Avonmouth, Bristol, UK), 2 μM NG25 (MedChem Express, Monmouth Junction, NJ), and 10 μM SB431542 (Tocris, Avonmouth, Bristol, UK). Following one week of growth, tumorspheres were viewed on the microscope and quantified. For limiting dilution assays, cells were serially diluted to be plated at 100, 50, 20, 10, 5, 2, or 1 cell(s)/well in 96-well plates. After one week of growth, wells were scored for the presence or absence of spheres. Extreme limiting dilution analysis was performed as previously described (Hu and Smyth, 2009), using software available at http://bioinf.wehi.edu.au/software/elda/.

Brain Slice Explantation and Tumorsphere Implantation

Coronal brain slices (250 μm thick) from postnatal day 10 Sprague-Dawley rat pups of either sex (Charles River, Wilmington, MA) were prepared and explanted in organotypic culture as previously described (Dunn et al., 2011). Animals were sacrificed in accordance with NIH guidelines and under Duke IACUC approval and oversight by the Lo Lab. Briefly, brain tissues were sliced in ice-cold artificial cerebrospinal fluid and plated in interface configuration atop culture medium (Neurobasal A medium supplemented with 15% heat-inactivated horse serum, 10 mM KCl, 10 mM HEPES, 100 U/ml penicillin/streptomycin, 1 mM sodium pyruvate, and 1 mM L-glutamine) semi-solidified in 0.5% reagent-grade agarose in 12-well plates. Brain slice explants were incubated under 5% CO_2 at 37 ºC for up to 8 days as indicated. Compound A or DMSO-only vehicle was added to culture medium at the

time of brain slice explantation. Small groups of GBM tumorspheres were implanted shortly thereafter by direct application to the upper surfaces of each brain slice. An epifluorescence stereomicroscope was used to obtain images through the course of experiments. ImageJ was used to quantify the area of GFP+ cells.

2.4 Results

NF-κB is preferentially activated in CD133+ cells of GBM explants

Given that NF-κB is not normally active in resting brain tissue, we first compared the growth of normal neural stem cells and astrocytes to that of human patient-derived GBM explant cultures. We treated cells with DMSO or with the selective IKKβ antagonist Compound A (Ziegelbauer et al., 2005) daily for 5 days and measured cell viability by MTS assay (Figure 2.1A). Results demonstrate that growth was impaired in the GBM explant cultures by NF-κB inhibition but not in the normal neural stem cell or astrocyte cultures, suggesting that NF-κB inhibition preferentially targets tumor cells over normal brain tissues. Since GBM was one of the first solid tumors in which CSCs were identified (Singh et al., 2003; 2004) and based on our previous work implicating NF-κB signaling in breast CSCs (Kendellen et al., 2013), we next asked if these findings could be explained by a role for NF-κB signaling in the growth of GSCs. To study the stem cell-like vs. non-stem cell-like subpopulation of glioblastoma cells, we isolated CD133+ vs. CD133- fractions from the human glioblastoma explant cultures using magnetic beads. Interestingly, the CD133+ cells exhibit elevated levels of p65 phosphorylation, consistent with increased or altered activity of the canonical NF-κB pathway (Figure 2.1B).

Inhibition of NF-κB reduces tumorsphere formation

We next examined whether NF-κB was important in tumorsphere formation in an *in vitro* test for stem cell-like activity. After isolation, CD133+ cells were plated at a low density and allowed to form spheroids for one week prior to quantification. Daily treatment with Compound A abrogated tumorsphere formation completely. Importantly, a single treatment with Compound A at the beginning of the experiment was sufficient to reduce tumorsphere formation, suggesting that even transient loss of NF-κB activity could affect stem-like activity (Figure 2.2A,B). In order to further address the effects on self-renewal, primary tumorspheres from the first week of growth were dissociated, re-plated, and then subjected to the same treatments. Treatment with Compound A also reduced secondary tumorsphere formation, again consistent with a role for NF-κB activity in the stem-cell fraction (Figure 2.2C). Finally, we assayed tumorsphere formation using a limiting dilution assay with a range of cell concentrations from 1-100 cell(s)/well. For both 7030 and GBM6 CD133+ cells, treatment with Compound A significantly reduced the ability of GBM stem cells to form tumorspheres (Figure 2.2D,E).

In order to validate the on-target activity of Compound A in inhibiting IKK, we repeated these assays using siRNA-mediated knockdown of IKKβ and/or p65. Knockdown of either protein resulted in a decrease in p65 phosphorylation, as well as a decrease in tumorsphere formation (Figure 2.3A-C). The inhibitory effects of p65 knockdown were confirmed in the limiting dilution assay consistent with previous results (Figure 3D). Taken together, these results strongly implicate a role for the NF-κB pathway in GSC propagation and self-renewal.

Multiple NF-κB subunits contribute to GSC maintenance

Up to this point, our studies have focused on the more highly studied canonical NF-κB pathway. We next sought to determine if the non-canonical NF-κB pathway, driven by a RelB-p52 dimer, could be contributing to GSC maintenance as well. Accordingly, GBM6 CD133+ cells were transfected with siRNAs targeting p65, p100/p52, or RelB. Each of these subunits produced a substantial decrease in tumorsphere formation in a limiting dilution assay (Figure 2.4A). Knockdown efficiency was confirmed through qPCR (Figure 2.4B). These results suggest that both the canonical and non-canonical NF-κB pathways contribute to GSC maintenance.

TAK1 activates the NF-κB pathway to promote GSC function

Next, we examined an upstream activator of the NF-κB pathway, transforming growth factor-β-activated kinase 1 (TAK1), which is known to activate the IKK complex following cytokine stimulation as well as in some oncogenic settings (Bosman et al., 2014; Melisi et al., 2011; Mizukami et al., 2002; Ninomiya-Tsuji et al., 1999; Takaesu et al., 2003). We first showed that use of two structurally distinct TAK1 inhibitors ((5Z)-7-oxozeanol and NG-25) decreased the expression of an NF-κB luciferase reporter in both GBM explant cultures (Figure 2.5A). In tumorsphere formation assays, (5Z)-7-oxozeanol and NG-25 treatment decreased tumorsphere formation in GBM 7030 CD133+ cells with a single treatment and to an even greater extent with daily treatment (Figure 5B). We then extended these studies in limiting dilution assays, finding that both siRNA against TAK1 and the TAK1 inhibitors resulted in significant decreases in tumorsphere formation (Figure 2.5C, D).

TGF-β is one source of NF-κB activation

As the cognate activator of TAK1 is transforming growth factor-β (TGF-β) itself, and as the TGF-β pathway has previously been implicated in regulating GBM CSCs (Ikushima et al., 2009; Peñuelas et al., 2009), we next investigated a potential link between TGF-β and NF-κB in the GBM setting. Studies showed that treatment with exogenous TGF-β led to an increase in Smad phosphorylation (as expected) and in p65 phosphorylation in GBM explant cultures (Figure 2.6A). Conversely, application of a TGF-βR1 inhibitor (SB431542) led to a decrease in p65 phosphorylation (Figure 2.6B). Additionally, the TGF-βR1 inhibitor induced a small but consistent decrease in luciferase activity from an NF-κB reporter in GBM explant cultures (Figure 2.6C). Together, these results suggest an autocrine/paracrine role for TGF-β in maintaining NF-κB signaling in these GBM explants culture. Use of the TGF-βR1 inhibitor in a tumorsphere assay leads to a significant decrease in tumorsphere formation in GBM6 CD133+ cells (Figure 2.6D). These results suggest that TGF-β can activate NF-κB signaling in these GBM explants. However, given the scale of these changes, it is likely only one of several sources of NF-κB activation, consistent with the pleiotropic nature of this pathway. Similarly, given the more drastic effects seen in the tumorsphere assay with the TGF-βR1 inhibitor, it is likely that the TGF-β pathway is also mediating additional factors involved in CSC biology.

Inhibition of the IKK/NF-κB pathway decreases glioblastoma growth ex vivo

Finally, we sought to validate the *in vitro* studies in a more biologically relevant setting, turning to an *ex vivo* organotypic brain slice preparation which has been used previously (Miao et al., 2014; Valiente et al., 2014). This methodology provided the

opportunity for higher throughput analysis, as well as a level of longitudinal imaging not typically available *in vivo*. For these experiments, neonatal rat brain tissues were sectioned into 250 μm coronal slices, then plated on top of an agar medium as previously described (Dunn et al., 2011). GBM6-GFP spheres were then engrafted onto the upper surfaces of these these brain slice explants shortly after slicing. A first round of imaging was then completed within a few hours of plating to establish a baseline for GBM tumor growth these brain slices. GBM tumor growth in the brain slices was then repeatedly imaged daily for up to eight days and the GFP-positive areas were quantified for each slice and on each day using ImageJ. Representative images show that implanted GBM tumorspheres progressively grow and invade the surrounding brain tissues over the course of the experiment (Figure 2.7A).

To examine a role for NF-κB in GBM growth within these ex vivo orthotopic xenografts, GBM6-GFP cells were transfected with either control or p65 siRNA 24 hours prior to tumorsphere formation and brain slice implantation. Quantification of tumorsphere cross-sectional areas indicated that transfection with p65 siRNA significantly inhibited the growth rate the implanted GBM tumorspheres over the eight days of the experiment (Figure 2.7B). Similarly, application of the IKKβ inhibitor to the implanted brain slice cultures also led to significant inhibition of GBM tumorsphere growth (Figure 2.7C, D).

2.5 Discussion

As with many other tumor types, glioblastoma is characterized by a hierarchical organization of cells, including a subpopulation of so-called cancer stem cells (or tumor-initiating cells). These cells promote tumor initiation and recurrence, drive invasion and metastasis, and demonstrate increased resistance to radiation and chemotherapy (Bao et al.,

2006; Cheng et al., 2011; Garcia et al., 2010; Liu et al., 2006; Singh et al., 2003; 2004). It is crucial to investigate the signaling pathways responsible for these phenotypic differences from the bulk of the tumor, both to establish insight into mechanisms that promote these cells and to potentially identify therapeutic targets for disease control. In this study, we demonstrate the involvement of the IKK/NF-κB pathway in the function of GBM CSCs. We identified a TGF-β/TAK1 axis as one source of NF-κB activation in these cells. TAK1 is a well-established activator of IKK and there is a precedent for cooperation between the TGF-β and NF-κB pathways in GBM (Song et al., 2012; Wang et al., 2015). Nonetheless, it is likely that other sources for NF-κB activation exist, such as cytokines, which utilize TAK1 as a mediator of NF-κB activation. Indeed, common genetic alterations seen in GBM (*PTEN* deletion, *EGFR* amplification and mutation, and monoallelic *NFKBIA* deletion) have all been connected to enhanced NF-κB activation (Asano et al., 2004; Bonavia et al., 2011; Bredel et al., 2011; Gustin et al., 2001; Rinkenbaugh and Baldwin, 2011; Tanaka et al., 2011; Yang et al., 2012; Ying et al., 2011). Our data from the *ex vivo* co-culture experiments show greater effects following IKK inhibition in the whole slice rather than just the cancer cells. Given the extensive number of cytokines and chemokines regulated by NF-κB, it is very likely that this pathway impacts the interactions between the tumor, microglia, and infiltrating immune cells, potentially related to the finding that GSCs promote tumor evasion via immunosuppression (WEI et al., 2010; Wu et al., 2010). Consistent with studies overall, Verma and colleagues showed that treatment with an NBD peptide impaired tumor growth in both a human glioma xenograft and a genetic glioma mouse model (Friedmann-Morvinski et al., 2016), focusing on inhibition of canonical NF-κB signaling through disruption of the IKK complex. Thus, the effects of IKK inhibition in GBM models may function, at least partly, at the level of

GSCs.

As a family of transcription factors, the NF-κB pathway is likely mediating a variety of downstream effects in GBM cells. We found that both the canonical and non-canonical branches contribute to GSC biology. The non-canonical pathway has been shown to have distinct functions in oncogenesis, such as driving growth and invasion of mesenchymal glioma and regulation of the mutant C250T *TERT* promoter (Lee et al., 2013; Li et al., 2015). Additionally, NF-κB is known to regulate target genes related to several GSC functions including survival (*BCL2*, *BCL2L1*), invasion (*IL6, IL8, CCL2, MMP2/3/9*), and resistance to therapy (*MGMT, TNFAIP3, TRADD*). Gene expression analysis showed NF-κB regulation of some of these targets, however neither the expression nor the NF-κB-dependency was limited to the CD133+ population of cells. It is likely that the effects on GSCs observed following NF-κB inhibition result from a combination of these and other genes. Given its central position in GSC signaling, the IKK/NF-κB pathway is proposed as a target for therapeutic intervention in glioblastoma targeting GSCs.

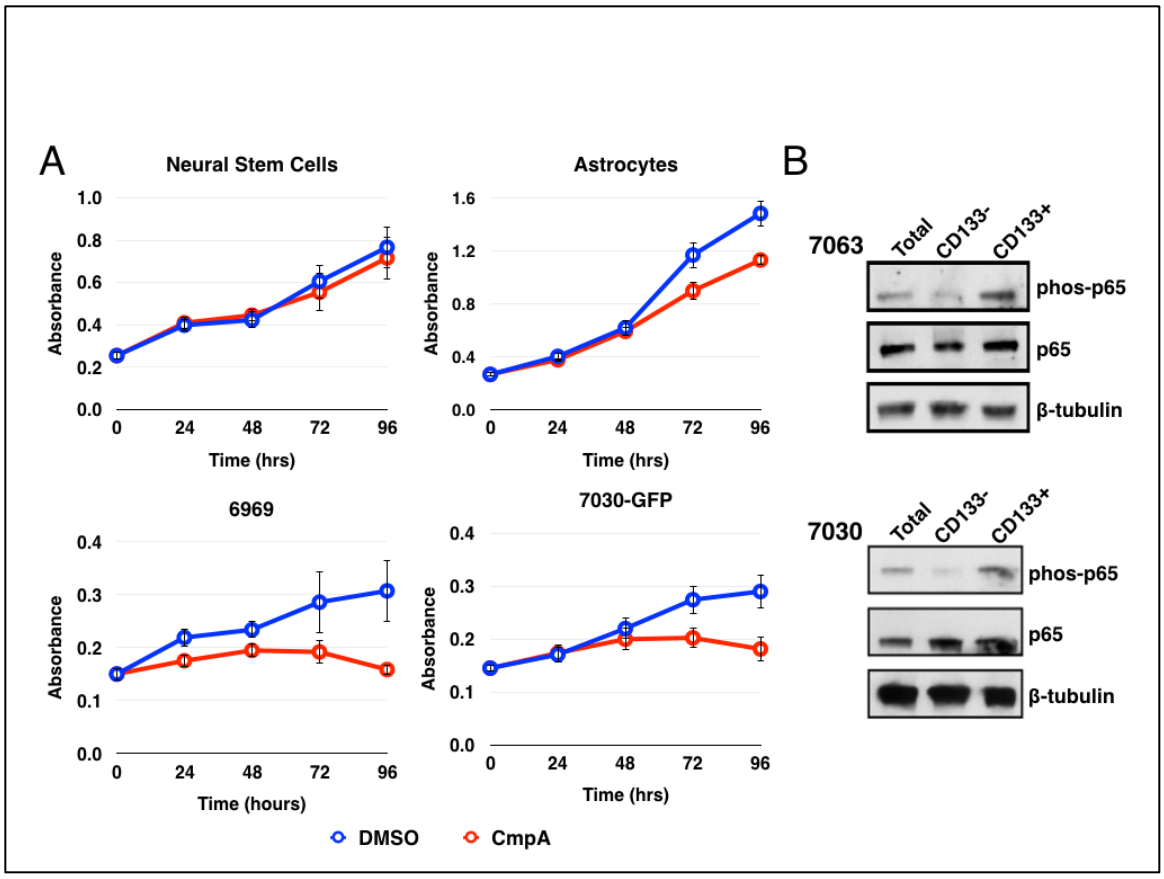

Figure 2.1 NF-κB is preferentially activated in CD133+ glioblastoma stem cells

(**A)** MTS assay using normal neural stem cells, astrocytes, or two GBM explants: 6969 and 7030. Cells were treated daily with DMSO or 5 μM Compound A and analyzed every 24 hours for 96 hours. Data are represented as the mean ± s.d. and are representative of three independent experiments. **(B)** Analysis of total cells, isolated CD133-, or CD133+ cells by immunoblot.

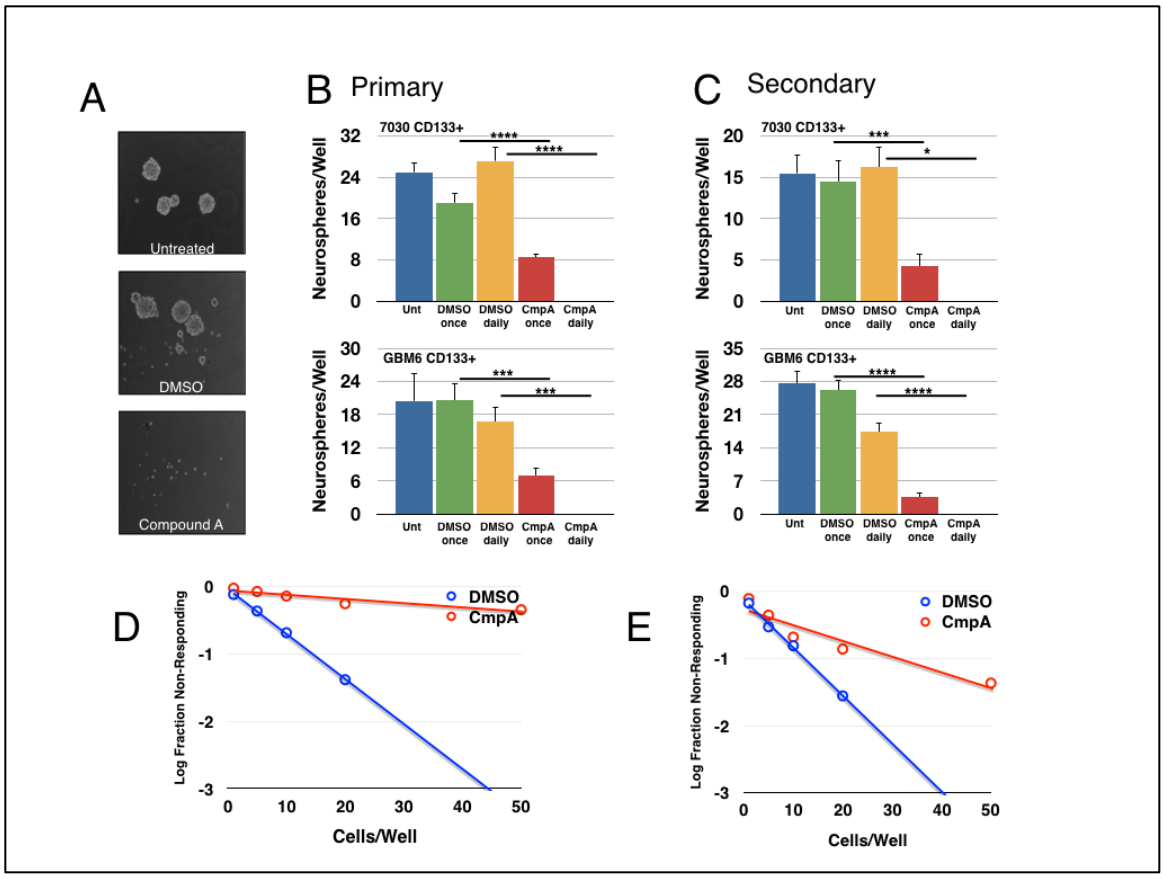

Figure 2.2 Pharmacological inhibition of the IKK/NF-κB pathway decreases neurosphere formation

7030 CD133+ cells plated for a neurosphere assay and treated as indicated (DMSO or 5μM Compound A, once or daily). After one week of growth, neurosphere formation was analyzed. **(A)** Representative images of neurosphere formation after daily treatment. **(B)** Quantification of primary neurospheres formed per well for two explants. Data are represented as mean ± SEM, ****p<0.0001, ***p<0.001, *p<0.05 by t-test. **(C)** Primary neurospheres were dissociated, replated, and treated again as indicated. Secondary neurosphere formation was quantified after another week of growth. Data are represented as mean ± SEM, ****p<0.0001, ***p<0.001, *p<0.05 by t-test. **(D, E)** Neurosphere formation

was measured through a limiting dilution assay with 7030 or GBM6 CD133+ cells plated at 100, 50, 20, 10, 5, or 1 cell(s)/well and treated with DMSO or 5μM Compound A (7030: n=48 wells/condition; $p=2.02x10^{-47}$; GBM6: n≥116wells/condition; $p=2.23x10^{-11}$).

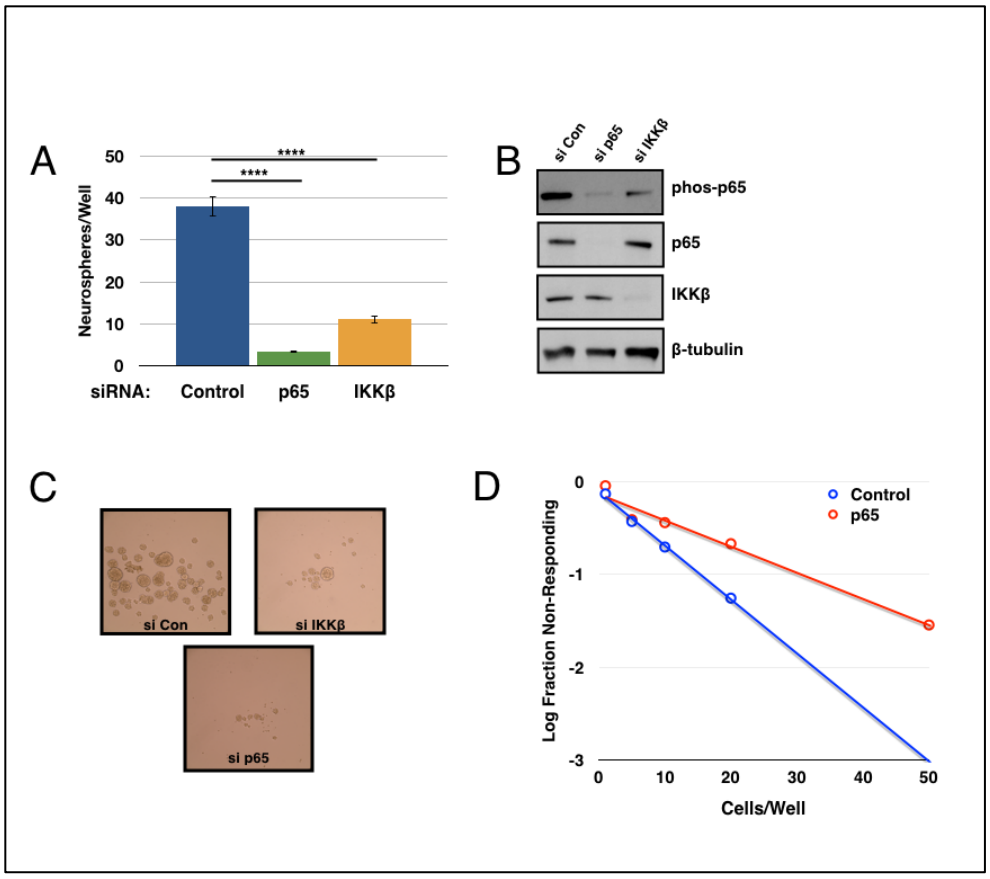

Figure 2.3 Genetic inhibition of the IKK/NF-κB pathway decreases neurosphere formation

7030 CD133+ cells were transfected with siRNA: control, p65, or IKKβ and then plated for neurosphere assays **(A)** Quantification of neurospheres/well after one week of growth. Data are represented as the mean ± SEM, ****p<0.0001 by t-test. **(B)** Transfected cells analyzed by immunoblot for p-p65, p65, IKKβ, and β-tubulin. **(C)** Representative images of neurosphere formation after daily treatment. **(D)** Neurosphere formation was measured through a limiting dilution assay with GBM6 CD133+ cells plated at 100, 50, 20, 10, 5, or 1 cell(s)/well following transfection with control or p65 siRNA ($n \geq 70$ wells; $p=1.76 \times 10^{-6}$).

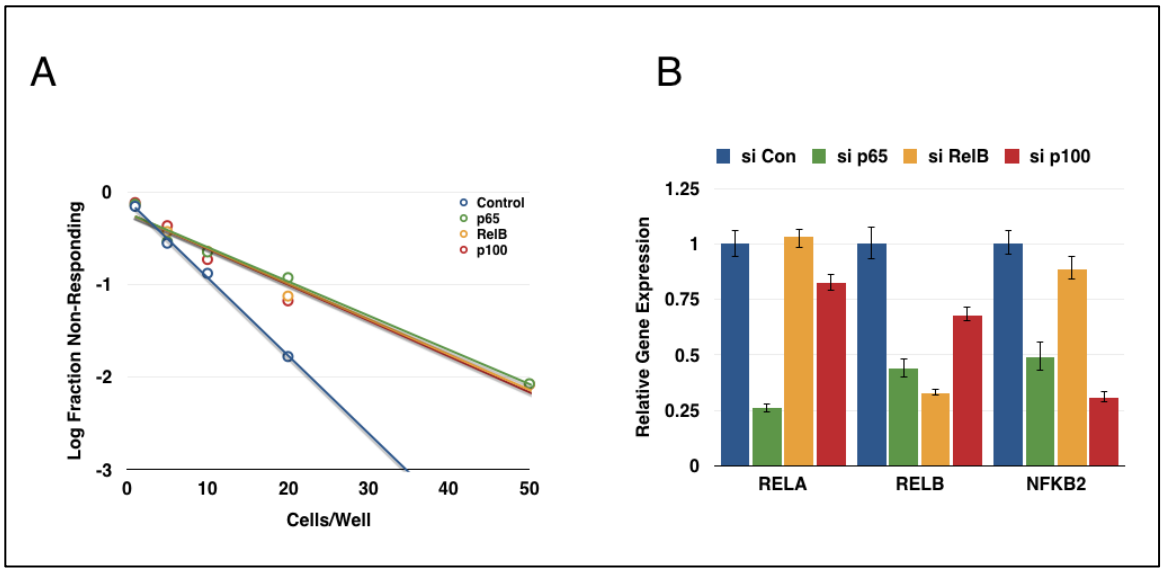

Figure 2.4 Multiple NF-κB subunits contribute to neurosphere formation

(A) GBM6 CD133+ cells were transfected with siRNA control or targeting p65, RelB, or p100. Subsequently, cells were plated out for limiting dilution assay at 100, 50, 20, 10, 5, or 1 cell(s)/well and scored for the presence or absence of neurospheres following one week of growth ($n \geq 95$ wells/condition; p65 versus control $p=2.26 \times 10^{-5}$; RelB vs. control $p=6.44 \times 10^{-5}$; p100 versus control $p=3.82 \times 10^{-5}$). **(B)** Quantitative real-time PCR to analyze expression of *RELA, RELB, or NFKB2* in siRNA-transfected cells normalized to *GUSB* expression.

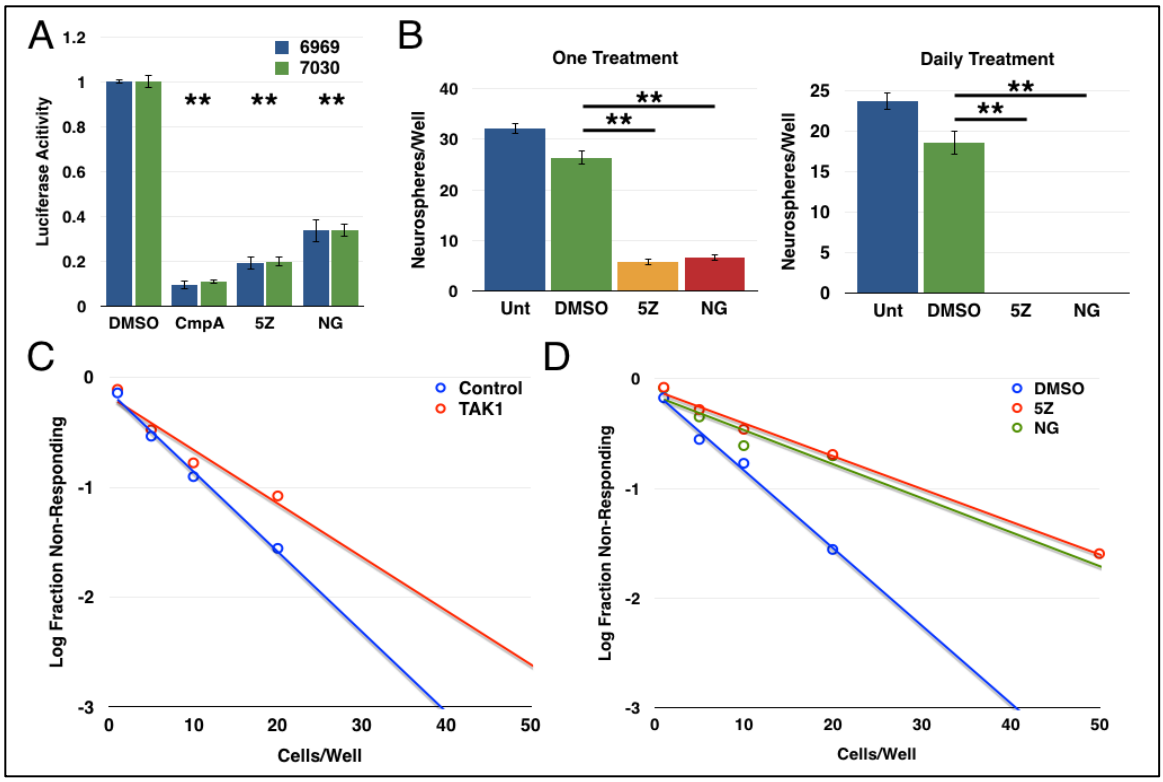

Figure 2.5 TAK1 activates the NF-κB pathway to promote glioblastoma stem cell function

(A) 6969 or 7030 cells were transfected with 3x-κB luciferase reporter, treated with the indicated inhibitors for 24 hours, then harvest and analyzed for luciferase activity (n=3; **p<0.0001 by t-test, error bars represent SEM) **(B)** Quantification of neurosphere formation in GBM6 CD133+ cells following treatment with either (5Z)-7-oxozeaenol or NG25 either once or daily. Data are represented as the mean ± SEM, **p<0.0001 by t-test. **(C)** Limiting dilution assay with GBM6 CD133+ cells transfected with siRNA control or TAK1 (n=72 wells/condition; p<0.05). **(D)** Limiting dilution assay following treatment of GBM6 CD133+ cells with structurally distinct TAK1 inhibitors: 2.5μM (5Z)-7-oxozeaenol or 2μM NG-25 (n≥90wells/condition; 5Z vs. DMSO $p=1.01x10^{-19}$, NG vs. DMSO $p=5.29x10^{-10}$).

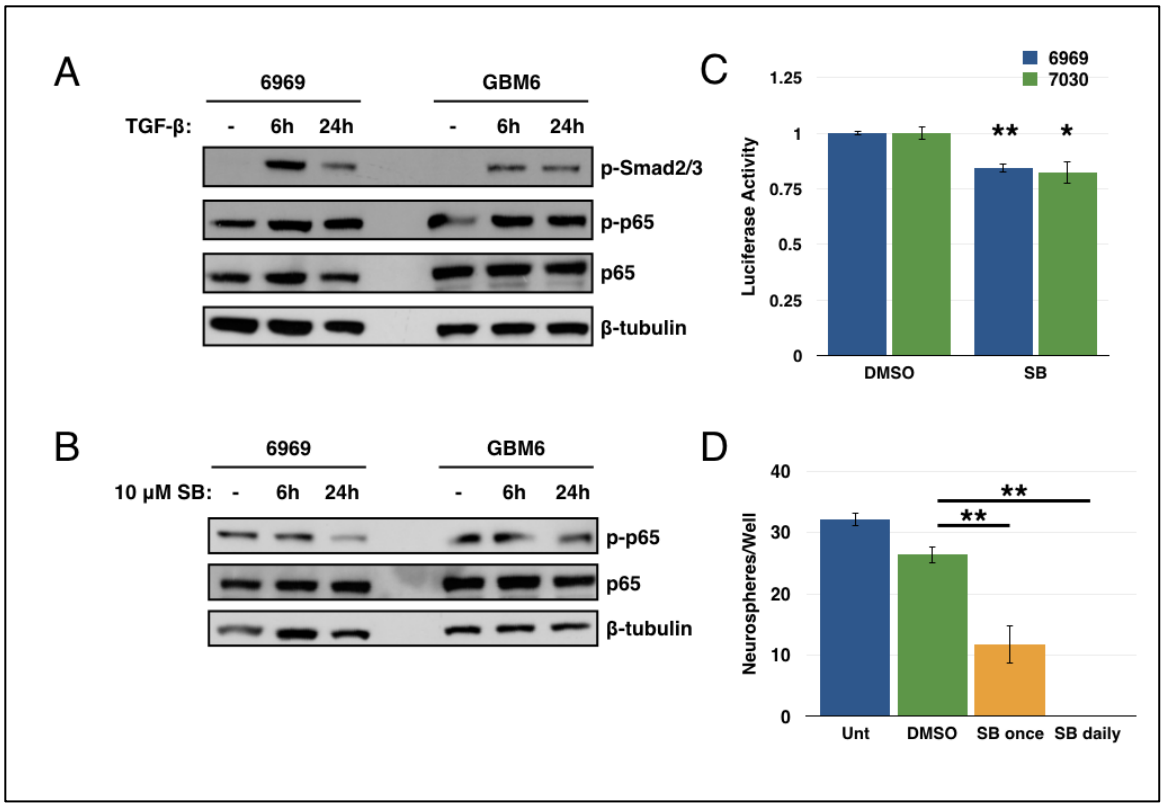

Figure 2.6 TGF-β is one source of NF-κB activation in GBM

(A) 6969 and GBM6 explants were stimulated with 10ng/mL TGF-β for 6 or 24 hours, then analyzed by immunoblotting for phosphorylation of Smad and p65. **(B)** 6969 and GBM6 explants were treated with 10 μM SB431542, a TGF-βR1 inhibitor, for 6 or 24 hours, then analyzed by immunoblotting for p65 phosphorylation. **(C)** 6969 or 7030 cells were transfected with 3x-κB luciferase reporter and treated with DMSO or 10 μM SB431542 for 24 hours, then harvested and analyzed for luciferase activity (n=3, **p<0.0001, *p<0.01 by t-test, error bars represent SEM). **(D)** Quantification of neurosphere formation in GBM6 CD133+ cells following treatment with 10 μM SB431542 either once or daily. Data are represented as the mean ± SEM, **p<0.0001 by t-test.

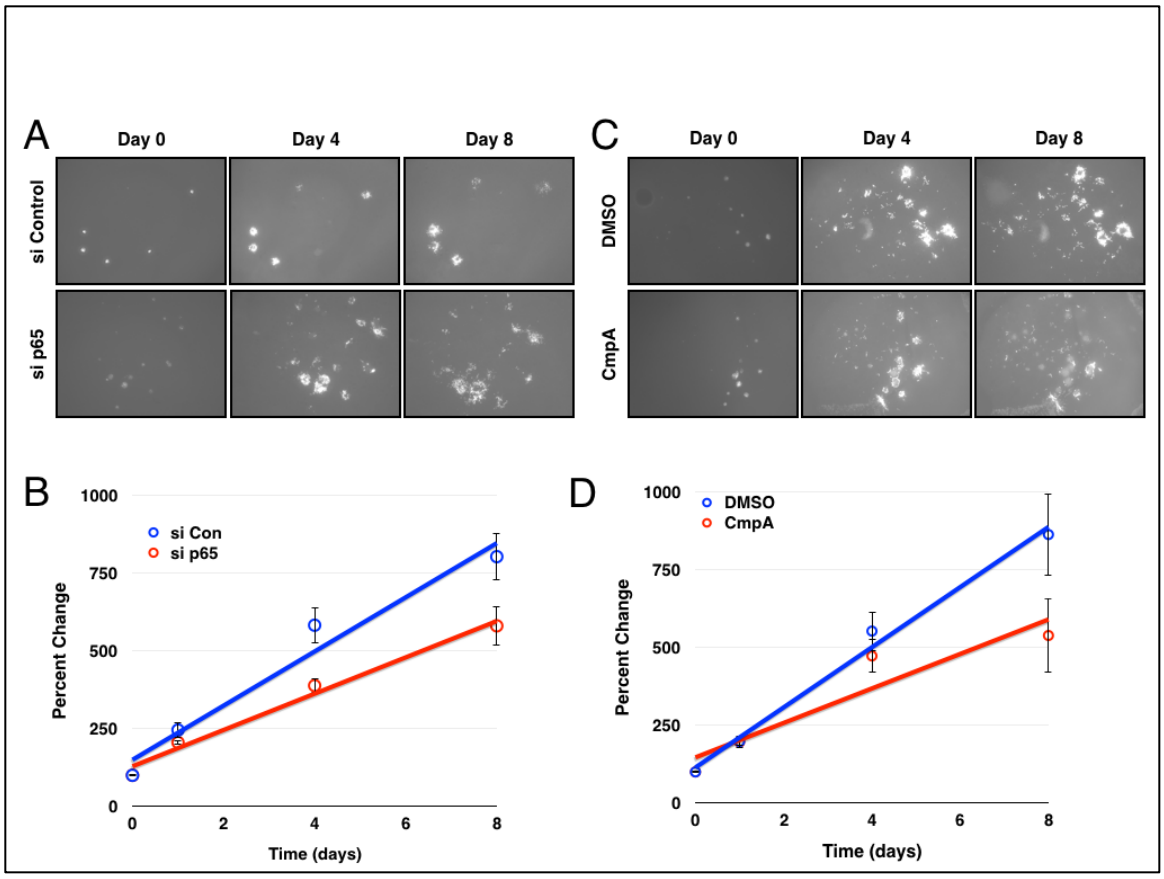

Figure 2.7 Inhibition of the IKK/NF-κB pathway decreases glioblastoma growth/survival *ex vivo*

(A) Images of GFP+ GBM6 cells following siRNA transfection and brain slice culture. **(B)** Average percent change in GFP+ area over the course of the experiment (n=14 for control, 13 for p65; $p<0.005$ by linear regression; error bars represent SEM) **(C)** Images of GFP+ GBM6 cells over the course of eight days of culture on rat brain slices following treatment with DMSO or Compound A. **(D)** Average percent change in GFP+ area over the course of the experiment (n=12; $p<0.01$ by linear regression; error bars represent SEM).

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CHAPTER III

NF-κB SIGNALING CONTRIBUTES TO PROLIFERATION AND INVASION IN CHORDOMA

3.1 Summary

Although chordoma is a slow growing tumor type, these tumors frequently invade locally and metastasize to distant sites. These characteristics contribute to tumor recurrence, which is a major source of mortality for these patients. Little work has been done to examine the invasive properties of these tumors to identify potential therapeutic targets. The NF-κB pathway is activated in many tumor types and mediates several aspects of cancer biology, including invasion. Here, we find that NF-κB is activated in human chordoma samples. Additionally, inhibition of NF-κB affects both proliferation of and migration by chordoma cell lines. Gene expression analysis shows decreases in IL6, IL8, and MMP9, which may be contributing to the phenotypes observed. This work demonstrates the involvement of NF-κB signaling in chordoma, suggesting that IKK inhibition may represent a novel therapeutic strategy.

3.2 Introduction

Chordomas are an incredibly rare tumor type with an annual incidence of 0.8 per million people (Smoll et al., 2013). They arise from remnants of the embryonic notochord and thus can be found anywhere along the spine, particularly at either the base of the skull or the sacrum. Though the tumors are relatively slow-growing, they present other clinical challenges as they are resistant to chemotherapy and radiation (Chugh et al., 2007; Forsyth et al., 1993). Additionally, they tend to recur and are both locally invasive and capable of metastasis, particularly to the lungs, bone, and liver. Current treatment generally includes radical surgery and high-dose radiation; however treatment is not standardized due to low patient volume and lack of molecular characterization. Distant metastases are responsible for up to 90% of cancer deaths, making it a critical topic for further investigation (Mehlen and Puisieux, 2006). Invasion poses a major challenge in treatment, as it can lead to destruction of adjacent normal tissue and makes it difficult to fully resect the primary tumor during surgery, especially given the proximity of crucial, delicate tissues near the base of the skull and along the spinal cord.

One aspect of invasion is degradation of the extracellular matrix (ECM) to allow the tumor cells to disseminate from the primary tumors. Many factors have been associated with this process, including the matrix metalloproteinases (MMPs). MMPs are able to break down a wide variety of substrates, including collagen, gelatin, fibronectin, and laminin. Epidermal overexpression of type I collagenase (MMP1) in a carcinogenesis model led to a significant increase in tumor incidence (D'Armiento et al., 1995). Conversely, deletion of MMP7 in the Min/+ model of colon cancer led to a marked decrease in tumor formation (Wilson et al.,

1997). Both of these results demonstrate involvement of the MMP family in promotion of cancer progression, but do not specifically involve invasive phenotypes. More recent studies have found that MMP9 expression is upregulated in invasive skin cancer lesions and MMP7 progressively accumulates as pancreatic tumors become metaplastic (Crawford et al., 2002; Kupferman et al., 2000). In chordoma, both MMP1 and MMP2 expression has been correlated with increased infiltration of bone as well as poor prognosis in patient specimens (Naka et al., 2004; 2008).

NF-κB is a family of transcription factors consisting of RelA/p65, RelB, c-Rel, p100/p52, and p105/p50 that operate as homo- or heterodimers and were originally discovered for their involvement in inflammatory and immune signaling. NF-κB dimers are sequestered in the cytoplasm by the inhibitory IκB proteins under basal conditions, but upon activation, the IKK complex phosphorylates IκBα, leading to its degradation. NF-κB is then able to translocate to the nucleus and regulate transcription of target genes. In addition to its role in inflammation and immune response, aberrant constitutive NF-κB activity has also been extensively tied to cancer biology. NF-κB target genes affect several hallmarks of cancer, such as increased proliferation and survival, suppression of apoptosis, and invasion (Bassères and Baldwin, 2006; Hanahan and Weinberg, 2000). For example, NF-κB has been shown to regulate transcription of MMP1 and MMP9 (Bond et al., 1998; He, 1996; Vincenti et al., 1998). Additonally, IKKα has been shown to promote invasion in Her2+ breast cancer (Merkhofer et al., 2010). NF-κB has not been well-studied in chordoma, but one report showed that both bortezomib and an IKK inhibitor showed efficacy in a chordoma xenograft model, but did not provide a mechanism for this effect (Trucco et al., 2013).

In this study, we explore the involvement of the NF-κB pathway in chordoma. We find that phosphorylated nuclear p65 is seen in patient samples, suggesting activation of the pathway in human chordomas. Knockdown of NF-κB subunits demonstrated that both p65 and p100 affected proliferation, suggesting involvement of both the canonical and non-canonical pathways. Use of the IKK inhibitor, Compound A, decreased wound closure in a scratch assay, demonstrating an effect on migration in these cells as well. Gene expression analysis shows a decrease in *IL6*, *IL8*, and *MMP9* expression, which could be mediating these effects on migration.

3.3 Materials and Methods

Cell Culture

UCH-1, JHC7, and MUG-Chor chordoma cell lines were obtained through the Chordoma Foundation (Durham, NC) and maintained in 4:1 IMDM:RPMI media (Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO) and 1% penicillin/streptomycin (Gibco, Carlsbad, CA) (Brüderlein et al., 2010; Rinner, 2011; Scheil et al., 2001). The cells were grown on plates coated with 0.1% gelatin.

Immunohistochemistry

Tissue sections obtained through the Chordoma Foundation Biobank (Durham, NC) were then stained with either hematoxylin and eosin or immunostained with phosphorylated p65 at serine 529 or total p65 (Cell Signaling Technology, Danvers, MA). Quantification of nuclear and cytoplasmic staining and intensity was performed with the UNC Translational Pathology Laboratory.

siRNA Transfection

Human siRNA targeting *RELA* (M-003533-02), *REL* (Hs00968436_m1), *NFKB2* (M-003918-02), or control #3 (D001201-03) was purchased from Thermo/Dharmafect (Lafayette, CO). DharmaFECT reagent 1 was used to transfect siRNA into cells according to the manufacturer's instructions. Six hours post-transfection, the media was changed on the cells. Cells were harvested 48-72 hours post-transfection for analysis by quantitative real-time PCR or Western blot.

3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium cellular proliferation assay.

Cells were transfected with siRNA six hours prior to seeding at 3000 cells per well in 96-well plates. For Compound A studies, cells were plated at 2000-3000 cells per well in 96-well plates with either DMSO or 5 μM Compound A. Fresh media containing the appropriate drug was replaced daily. At each time point, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) compound (Promega, Madison, WI) was added and absorbance was read at 490 nm on a Versamax Microplate Reader (Molecular Devices, Sunnyvale, CA).

Quantitative real-time PCR

RNA extracts were obtained from cells using the RNeasy Plus Kit (Qiagen, Hilden, Germany). Two micrograms were reverse transcribed using random primers and MMLV reverse transcriptase (Invitrogen, Carlsbad, CA). Real-time PCR was performed using Taqman Gene Expression Assay primer-probe sets for *GUSB* (Hs99999908_m1), *RELA*

(Hs00153294_m1), *RELB* (Hs00232399_m1), and *NFKB2* (Hs01028901_g1) and relative quantification was determined using the $\Delta\Delta C_t$ method.

Wound Healing Assay

Cells were plated at a density of 250,000 cells on gelatin-coated 35mm dishes with a glass cover slip in the center. Samples were pre-treated with DMSO or 5 μM Compound A for 16 hours. Subsequently, the plates were scratched with a 20 μL pipette tip and received fresh media with appropriate drug added. Images were acquired hourly over 24 hours and analyzed for wound closure using ImageJ.

3.4 Results

NF-κB is activated in human chordoma and mediates cell proliferation

Many tumor types demonstrate aberrant, constitutive NF-κB activation, however chordoma has not previously been shown to have active NF-κB. Human chordoma samples were fixed and paraffin embedded, then analyzed by immunohistochemistry. These samples showed positive staining for phosphorylated p65 localized to the nucleus, consistent with NF-κB activation (Figure 3.1). One matched set of normal adjacent psoas muscle and chordoma was provided for immunostaining. The tumor showed more p65-positive nuclei and a stronger intensity of staining (as indicated by the H-score) than the normal tissue (Figure 3.2). Finally, another set of human chordoma samples was immunostained for p65, showing a range of p65-positive nuclei from 10%-80% (Figure 3.3). The heterogeneity of

staining suggest that canonical NF-κB activation may be limited to a subset of chordoma cases.

Given evidence that there is increased NF-κB signaling in chordoma, we sought to determine what processes NF-κB might be regulating in these cells. We tested the effect of Compound A treatment on three chordoma cell lines (UCH-1, JHC7, and MUG-Chor) by MTS assay. After 120 hours of growth, all three cell lines showed modest decreases in proliferation in the presence of Compound A (Figure 3.4A-C). In order to complement the pharmacological studies with genetic inhibition, the UCH-1 cell line was transfected with siRNAs against several subunits: p65, c-Rel, and p100, then monitored for proliferation and viability through MTS assay. After 96 hours of growth, cells transfected with either p65 or p100 showed decreased absorbance when normalized to the growth of control cells (Figure 3.4D). Interestingly, these results show that both the canonical and non-canonical NF-κB pathways contribute to this phenotype.

NF-κB signaling contributes to migration of chordoma cells

Since chordoma is characterized as a relatively slow-growing but invasive tumor type, we next asked if NF-κB signaling mediated a migratory phenotype in chordoma cell lines. UCH-1, JHC7, and MUG-Chor cell lines were plated for a wound healing assay and pre-treated with either DMSO or the IKK inhibitor Compound A for 16 hours. Then, the plates were scratched and received fresh media containing the appropriate inhibitor. Images were acquired hourly over a 24-hour span and the area of the scratch was quantified. Though the cells were slowly moving, preventing full closure of the wound in the observed time span, a difference between DMSO and Compound A-treated cells could still be detected

(Figure 3.5). Interestingly, when compared to the MTT results (Figure 3.4), the change in the wound healing assay precedes significant changes in the MTT, suggesting these effects are primarily due to migratory properties and not a proliferation defect.

In order to begin to investigate potential mediators of chordoma migration and potentially invasion, we examined a set of known NF-κB target genes known to have ties to these processes. The three chordoma cell lines were treated with Compound A for 24 hours, then RNA was collected for quantitative real-time PCR analysis. The cytokines IL-6 and IL-8 were both significantly downregulated (Figure 3.6). Matrix metalloproteinases are known to break down the extracellular matrix, which contributes to tumor invasion. Here, MMP9 but not MMP2 or MMP3 were found to be downregulated in these cells (Figure 3.6). While multiple MMPs are associated with invasion in many tumor types and models, MMP9 may be particularly important in chordoma.

3.5 Discussion

Since chordoma is such a rare tumor type, it has not been well-studied. While relatively slow growing, these tumors tend to recur, invade locally, as well as metastasize to distant sites. Invasion and metastasis impair treatment of the primary tumor and frequently contribute to tumor recurrence, poor prognosis, and ultimately mortality. Chordomas are highly resistant to radiation and chemotherapy. Thus, current treatment of chordoma is mainly reliant on surgical resection, meaning that invasion is an even bigger problem. As such, it is crucial to better understand the underlying biology of these processes in chordoma cells to improve patient treatment and outcomes.

The NF-κB pathway is known to be constitutively activated in many tumor types and regulates a wide variety of target genes. Here, we observed strong nuclear localization of phosphorylated and total p65 in multiple chordoma cases, as well as increased immunostaining when compared to the normal adjacent tissue. NF-κB signaling is associated with many of the biological processes that drive tumor formation and progression, including invasion. In this study, we demonstrate activation of NF-κB in human chordoma samples. Compound A treatment or knockdown of NF-κB subunits shows that loss of either p65 or p100 decreases chordoma cell viability, suggesting involvement of both the canonical and non-canonical branches. Use of the IKK inhibitor, Compound A, decreases the migratory capabilities of chordoma cell lines, as demonstrated in a wound closure assay. Gene expression analysis showed a decrease in expression of *IL6*, *IL8*, and *MMP9* following IKK inhibition, consistent with their involvement in regulating invasion. More work is needed to determine if those genes are directly responsible for the effects seen in the wound closure assay or if other genes may also be contributing. It would also be useful to delineate the roles for canonical versus non-canonical subunits in these cells, as both contribute to proliferation, but have not yet been tested with respect to invasion. Ultimately, investigation in genetic or xenograft models in mice will provide even more insight as to whether NF-κB could be a useful therapeutic target in chordoma. The process of invasion involves many components besides the tumor cells (i.e. extracellular matrix, blood vessels, infiltrating immune cells) and they would be better recapitulated in animal models than in cell culture.

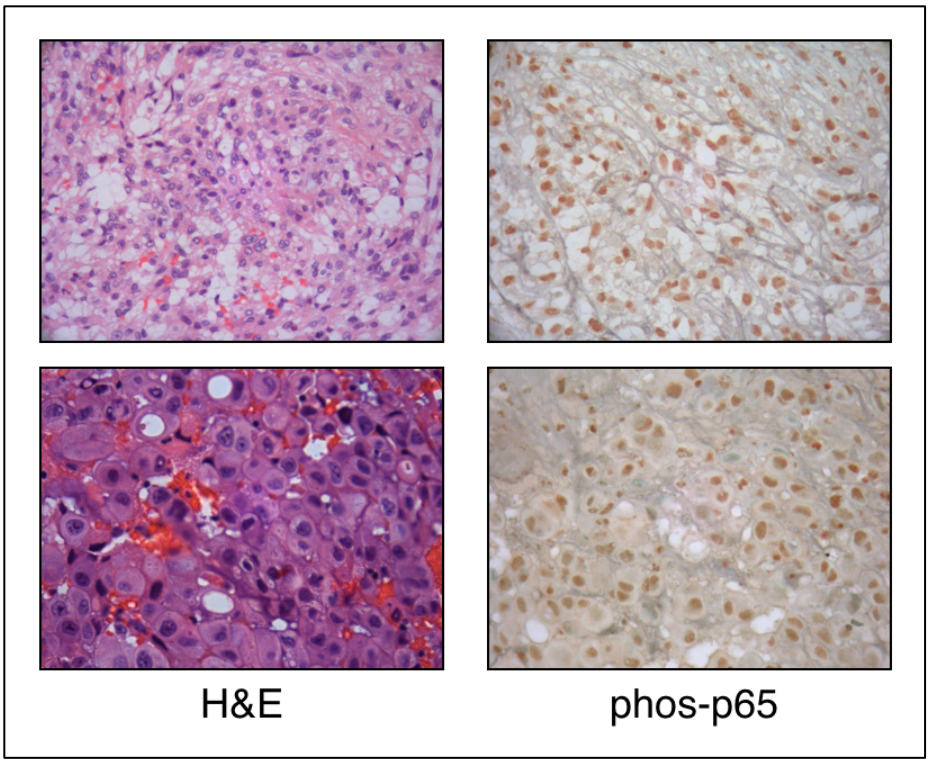

Figure 3.1 NF-κB activation in human chordoma samples

Human chordoma samples were fixed in formalin and embedded in paraffin, then stained by immunohistochemistry. Samples on the left were stained with hematoxylin and eosin (H&E, left), while adjacent sections were immunostained for phosphorylated p65 (right). The phosphorylated p65 staining is localized to the nucleus, consistent with NF-κB activation.

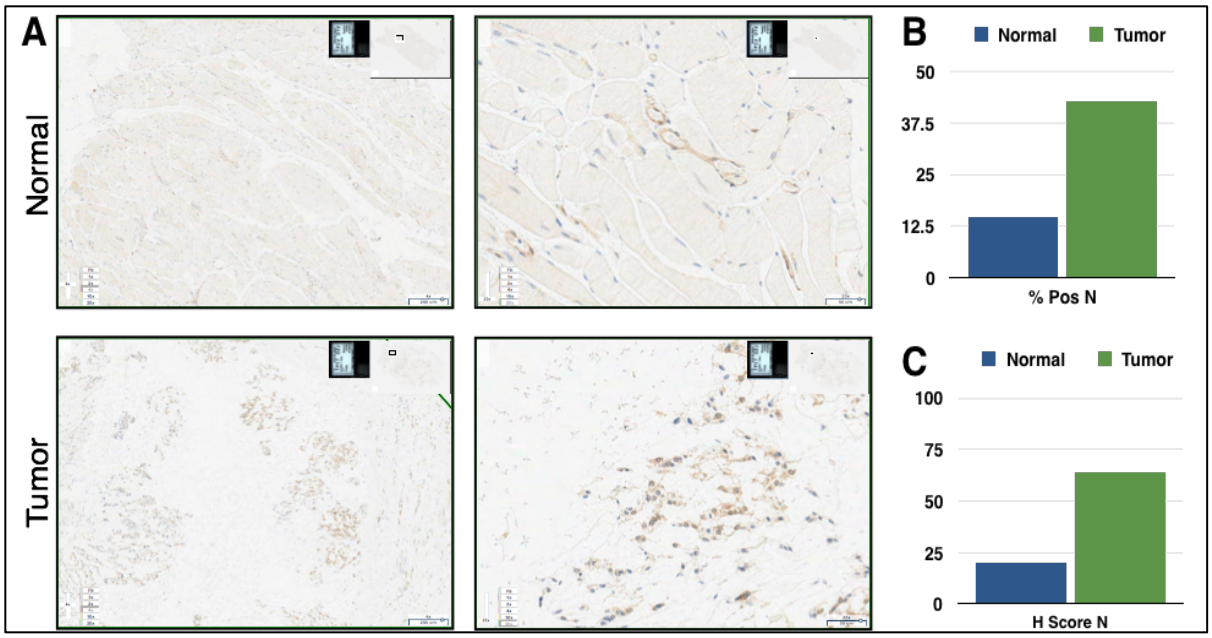

Figure 3.2 Increased nuclear localization of p65 in chordoma samples

(A) Immunostaining of histological sections of a chordoma sample or adjacent psoas muscle with p65 at 4x (left) or 20x (right) magnification (B) Quantification of percentage of cells with positive nuclear staining (C) H-score for nuclear p65 staining, which takes staining intensity into account

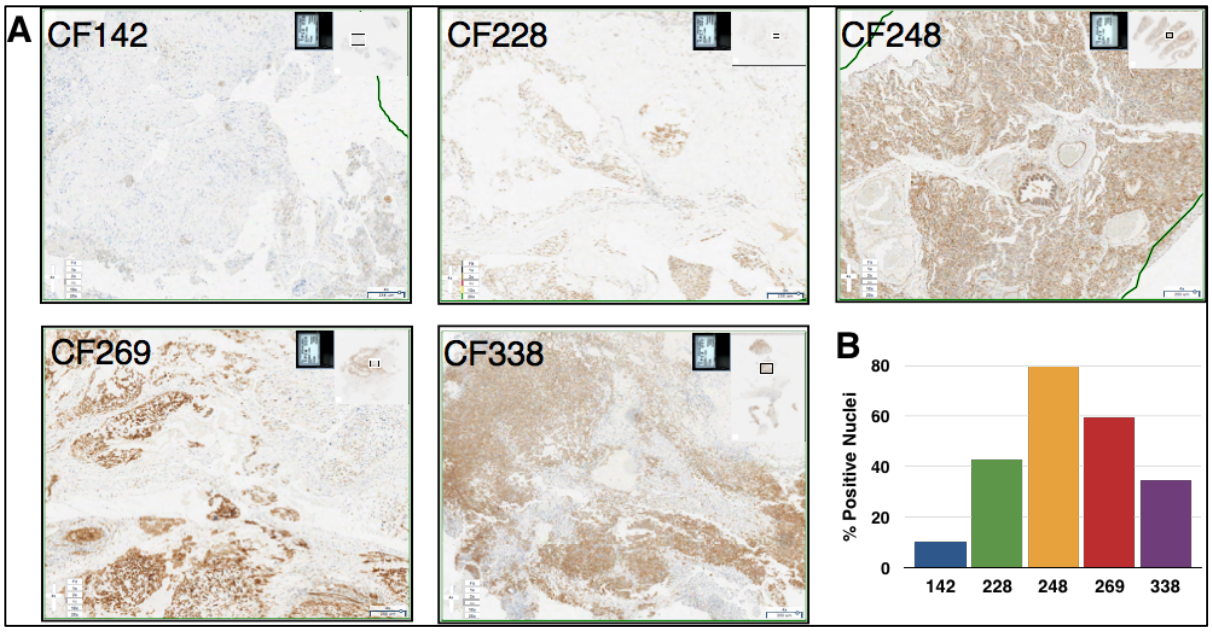

Figure 3.3 Heterogeneous p65 staining across chordoma cases.

(A) Immunostaining of histological sections of chordoma cases with p65 at 4x magnification

(B) Quantification of percentage of cells with positive nuclear staining

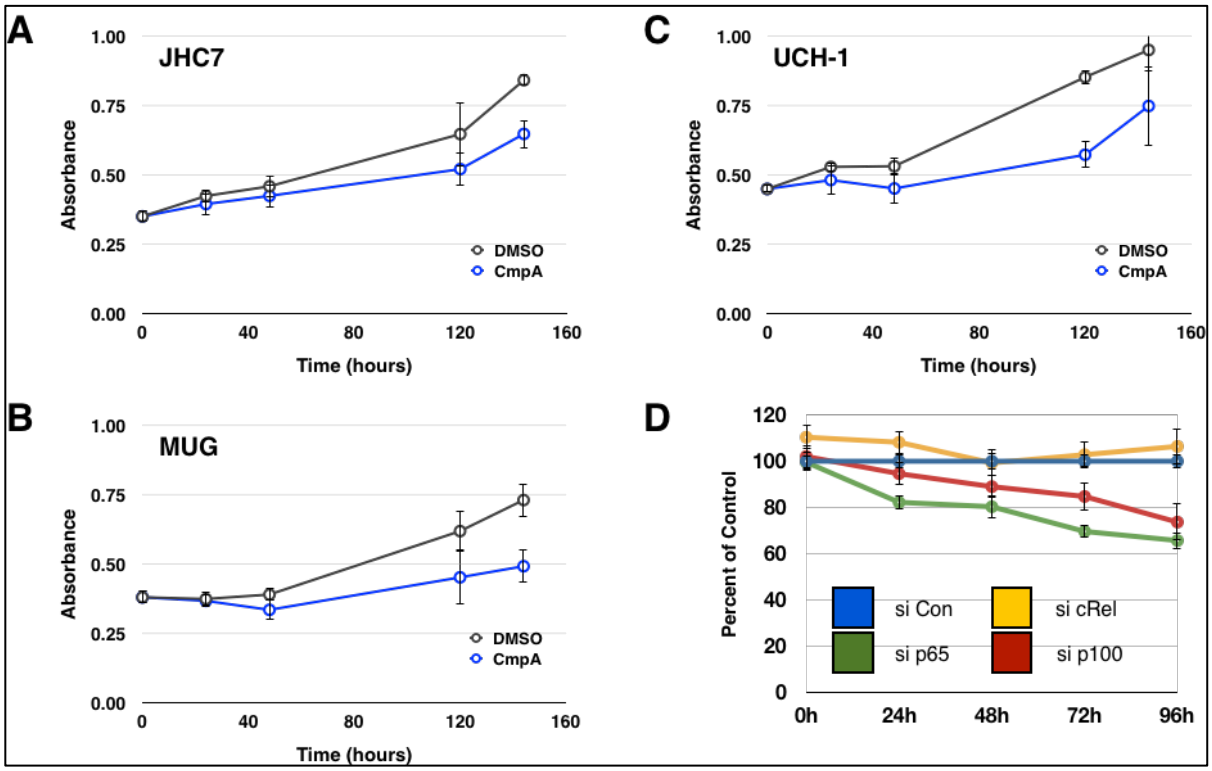

Figure 3.4 Inhibition of NF-κB impairs proliferation of chordoma cell lines.

(A-C) MTT assay of JHC7, MUG, and UCH-1 cell lines treated with DMSO or 5 μM Compound A (D) MTT assay of UCH-1 cells following siRNA transfection for canonical and non-canonical NF-κB subunits, p65, cRel, or p100. Error bars represent mean ± s.d.

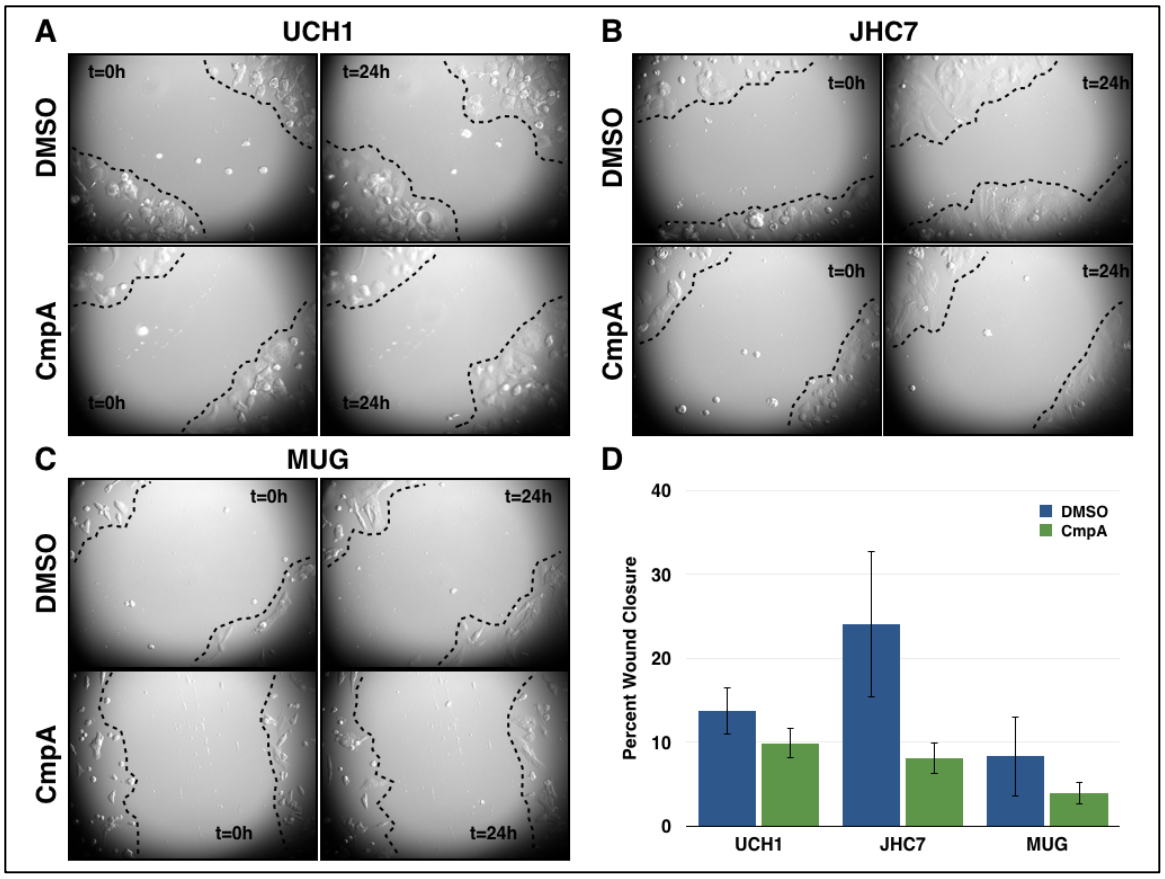

Figure 3.5 IKK inhibition impairs chordoma cell invasion

Chordoma cell lines were pre-treated with DMSO or 5μM Compound A for 16 hours. Then, the plates were scratched and fresh inhibitor was administered. Images were collected hourly for 24 hours for UCH-1 **(A)**, JHC7 (**B)**, and MUG **(C)**. **(D)** Quantification of percent wound closure over 24 hour time period using ImageJ. Errors bars represent mean ± s.d.

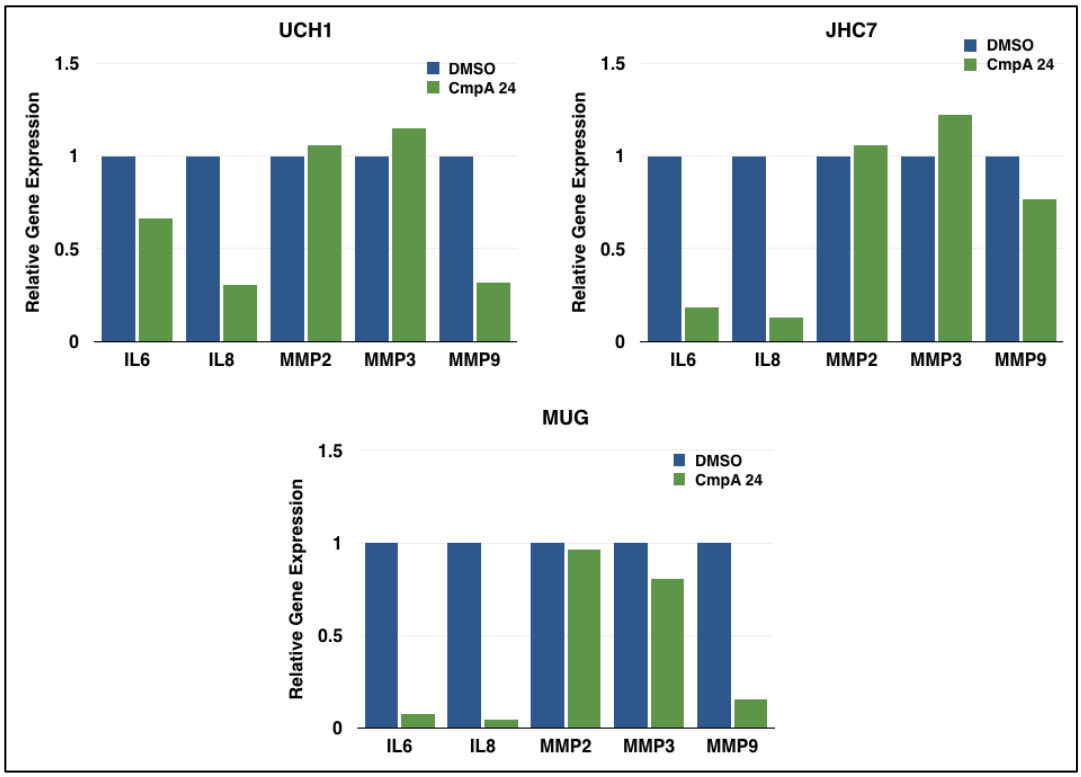

Figure 3.6 IKK inhibition decreases expression of *IL6*, *IL8*, and *MMP9*

Each of the chordoma cell lines (UCH-1, JHC7, and MUG) was treated with Compound A for 24 hours, then RNA was harvested. Following cDNA conversion, gene expression was analyzed. *IL6* and *IL8* were both decreased in all cell lines. Meanwhile, *MMP9*, but not *MMP2* or *MMP3* was downregulated after Compound A treatment.

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CHAPTER IV

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions and Future Directions

NF-κB is a pleotropic family of transcription factors that regulate a diverse set of target genes that subsequently mediate a wide variety of biological processes. While initially discovered through its involvement with immune and inflammatory signaling, the NF-κB pathway has since been implicated in the oncogenesis and tumor progression through a number of mechanisms and across many model systems and tumor types (Baldwin, 2012; DiDonato et al., 2012; Hayden and Ghosh, 2008). In the previous chapters, we have specifically explored roles for NF-κB signaling in glioblastoma and chordoma through effects on cancer stem cells and invasion.

First, we sought to determine if NF-κB was important to the cancer stem cell population in glioblastoma explants. Many studies investigate CSCs due to their involvement in driving tumor initiation, recurrence, and metastasis (Reya et al., 2001). Additionally, they

have been shown to be more resistant to radiation and chemotherapy (Bao et al., 2006; Beier et al., 2007), the current main treatment modalities in oncology. With these characteristics in mind, our understanding of tumor organization shifts from a relatively homogeneous and genetically similar group of cells to a hierarchically organized and heterogeneous tumor, which more faithfully recapitulates what has been observed in human tumors. This model suggests that dissecting the biology of CSCs could allow them to be targeted by novel therapies, either to kill these cells specifically or to drive them out of the CSC niche and increase the susceptibility to other treatments, which are currently less effective. We find that NF-κB is preferentially activated in the CD133+ subset of GBM explants (Figure 2.1) and that inhibition of this pathway through either pharmacological or genetic means decreases the self-renewal capacity of these cells (Figures 2.2 and 2.3).

Though most of the work focused on canonical NF-κB signaling, there is also evidence that the non-canonical pathway is involved in these cells (Figure 2.4). However, this should be more fully investigated to determine the degree of overlap between the two branches of NF-κB signaling in this context. All of the subunits bind the same consensus NF-κB sites to regulate target gene expression, yet they are known to regulate both common and distinct sets of target genes. Traditionally, there are distinct stimuli that can specifically activate each branch individually (i.e. TNF for canonical; CD40 for non-canonical), however this distinction is less clear in the case of oncogenic signaling, where numerous upstream factors can lead to NF-κB activation. In our studies, we did not see a different amount of p100/p52 processing between CD133+ and CD133- as compared to the preferential p65 phosphorylation observed. Nonetheless, knockdown of p65, RelB, or p100 in CD133+ cells

all produced a similar decrease in self-renewal in limiting dilution assays, perhaps suggesting similar roles for all of these subunits.

Future work needs to further investigate the NF-κB target genes that are mediating the phenotypes observed in these studies. Known NF-κB targets such as IL-6, IL-8, and A20 have been associated with GBM pathogenesis. Microarray analysis of CD133+ cells treated with Compound A also demonstrated NF-κB regulation of additional targets, such as CCL2 and MAP3K8 (data not shown). Individually, these genes do not appear to have significant affects on neurosphere formation, nor is their expression biased to the CD133+ population over CD133- cells. It is possible that the most important factors downstream of NF-κB in CSCs have yet to be identified. More likely, NF-κB is regulating several genes, all of which contribute partially to the phenotype, but it is only when the whole unit is affected that an effect is observed in the CSCs. To better understand this, an NF-κB signature would need to be defined, ideally specifying genes that are either more highly expressed in CD133+ cells or differentially regulated from the CD133- fraction.

None of this is meant to imply that there is not a role for NF-κB in the CD133- cells. The data from the MTT assay in Figure 2.1 shows a decrease in proliferation following Compound A treatment in unfractionated GBM cells. Historically, NF-κB signaling has been implicated in pro-survival and anti-apoptotic functions in GBM cells, which were not specific to CSCs. Some evidence suggests that the non-CSCs are crucial to providing support to the CSCs, either through paracrine secretion of cytokines (i.e. IL-6) or direct interaction between the cells (i.e. Notch ligands and receptors). We could imagine an attractive model where NF-κB activation in the non-CSCs leads to cytokine production, activating NF-κB in the CSCs to regulate a different gene signature to mediate stemness and self-renewal.

Another question that has yet to be fully investigated involves the plasticity between these CSC and non-CSC compartments. In GBM, at least three different types of CSCs have been described, which vary in their marker expression and capacity for self-renewal and tumor initiation (Chen et al., 2010). In breast cancer, culture of isolated stem-like, luminal, and basal cells led to a stochastic reversion back to the original composition of the cell lines (Gupta et al., 2011). These models arrive at different conclusions about whether the heterogeneous composition of the samples are driven by hierarchical organization or stochastic transitions; however both suggest that cells are moving between multiple cell states, a phenomenon that is rarely investigated. In order to better tackle these questions, we need better methods to track individual cells over time within a population, but with innovations to imaging, sequencing, and flow cytometry methods, these types of experiments are becoming more and more feasible.

One source of NF-κB activation in these cells is from a TGF-β/TAK1 signaling axis (Figures 2.5 and 2.6). However, these results showed a discrepancy between the degree of change in NF-κB activation and the decrease in neurosphere formation following treatment with a TGF-βRI inhibitor. These results suggest that TGF-β is only one source of many for NF-κB activation in these cells. Future work should further investigate upstream mechanisms that ultimately meet at the NF-κB pathway. Previous research has already tied EGFR and the ligand-independent mutant, EGFRvIII, with NF-κB, but there are probably still many others to be described. Potentially, this could provide evidence that NF-κB is a critical signaling node across GBM, regardless of subtype or mutational status. One example of this possibility came with the identification of monoallelic NFKBIA deletions in a subset of GBMs that were

mutually exclusive with EGFR-amplified tumors (Bredel et al., 2011; Rinkenbaugh and Baldwin, 2011).

We also investigated the involvement of NF-κB signaling in chordoma. Low incidence and a lack of model systems has limited the molecular characterization of chordomas, although recent efforts by the Chordoma Foundation have validated several cell lines and animal models. We found phosphorylated p65 localized to the nucleus in human chordoma samples, indicating activated NF-κB signaling in these tumors (Figure 3.1). In a wound healing assay, chordoma cell lines treated with Compound A did not close the wound as efficiently as the control cells (Figure 3.3). Subsequent gene expression analysis showed that the cytokines IL-6 and IL-8, as well as MMP9 but not MMP2 or MMP3 were downregulated following Compound A treatment (Figure 3.4). These data demonstrate a role for NF-κB signaling in the invasive capabilities of chordoma cell lines, consistent with previous work implicating the MMPs in contributing to chordoma invasion (Naka et al., 2004; 2008). Decreasing the invasion of chordomas would have substantial therapeutic benefit. Local invasion of the primary tumor leads to destruction of adjacent normal bone tissue and makes complete surgical resection of the tumor much more difficult, especially since these tumors localize to the base of the skull and the spinal column.

Another crucial characteristic of chordoma is the resistance to radiation and conventional chemotherapies. Previous work has linked NF-κB to similar phenotypes in many cancers, but these questions have not been examined in the context of chordoma (Bednarski et al., 2009; Chen et al., 2008; Cusack et al., 2001; Wang et al., 1999; 1996). Thus, it would follow that NF-κB could be operating in a similar manner in these tumors, potentially through regulation of pro-survival and anti-apoptotic genes. Future experiments

can combine IKK inhibition with chemotherapy to determine if there is increased sensitivity and cell death compared to either treatment individually.

One overarching application of the findings presented here is the utility of IKK or NF-κB inhibition as a therapeutic target. Transcription factors simultaneously represent both potent and difficult drug targets. A transcription factor generally regulates expression of several genes simultaneously to promote some phenotype within the cell, so targeting the transcription factor can have a widespread effect. Conversely, such inhibition can also affect more genes than just those of interest and have negative off-target effects. This paradigm can be specifically applied in the case of IKK and NF-κB inhibition. As detailed throughout this dissertation, NF-κB contributes to wide variety of cancer processes and *in vitro* inhibition has impaired self-renewal, proliferation, and invasion. Nonetheless, there are major concerns that systemic and sustained NF-κB inhibition would lead to immunosuppression and an inability to respond to inflammatory stimuli. One approach has been to develop IKKβ-specific small molecule inhibitors, which should significantly impair canonical signaling but leave non-canonical, IKKα-driven signaling intact (DiDonato et al., 2012). Another approach is the use of peptide inhibitors against the NEMO-binding domain, again targeting the canonical IKK complex without affecting non-canonical signaling. These inhibitors show promising results in pre-clinical animal models of cancer (Bassères et al., 2014; Friedmann-Morvinski et al., 2016; Xue et al., 2011). It may also be possible to deliver these inhibitors in a more targeted manner against the tumor, which could spare the systemic effects on the immune system. For example, NF-κB is not particularly active in the normal brain, so off-target tissue effects may be minimal in this setting. Ultimately, clinical trials will be needed to investigate whether

there is a therapeutic window in cancer treatment, where the anti-tumor effects can be balanced with any negative impact on the immune system.

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