NF-κB INDUCING KINASE (NIK) IS AN INDUCER OF GLIOMA INVASION

A Thesis

by

KASSANDRA NICOLE MCFADDDEN

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Committee Chair,	Raquel Sitcheran
Committee Members,	Vishal Gohil
	Westin Porter
	Farida Sohrabji
Head of Department,	Warren Zimmer

December 2018

Major Subject: Medical Sciences

ABSTRACT

Glioblastomas (GBMs) are a malignant subtype of brain tumor hallmarked by a high proliferation rate, cell migration, and aggressive invasion into healthy surrounding brain tissue. The extremely invasive phenotype of GBMs contributes exponentially to the high tumor recurrence and low median survival rate of patients. Our lab has demonstrated that NF-κB Inducing Kinase (NIK/MAP3K14), the primary driver of the noncanonical NF-κB pathway, is essential for glioma invasion and tumor formation. We have shown that abolishing NIK attenuates invasion, while cells with low NIK expression are significantly less invasive. Recent observations have shown that specific induction of NIK mRNA occurs in response to stimulation with pro-invasive cytokines (e.g. TNF-like Weak inducer of apoptosis). Here, we address the effects of increased NIK mRNA expression on glioma cell invasion and the effects of inhibition of NIK. We also examine NIK-dependent paracrine effects that promote a leader cell phenotype by enhancing the invasion potential of low invasive cells.

DEDICATION

I would like to dedicate this thesis to my family for their constant love, support, and encouragement.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Dr. Raquel Sitcheran, for her mentorship and guidance throughout my graduate studies. I would also like to thank my master's committee members, Dr. Vishal Gohil, Dr. Farida Sorhabji, and Dr. Weston Porter. I would like to express my gratitude to each of you for your guidance and feedback throughout this entire process, I greatly appreciate it.

I also like to thank all the current and former members in Dr. Sitcheran's lab, Linda Herrera de Lechuga, Dong Whan Lee, Sowndharya Ravi, Jung Jiung, Zoe Alaniz, Michael Kamradt, Kathryn Pflug, Marianne Best, Asmita Upreti, and Fred Price for their insight, mentorship and support. I would especially like to thank Linda for her guidance, and patience when I first joined the lab, and Zoe for her willingness to lend a hand whenever necessary.

Finally, I am deeply thankful for my parents and brother, who have encouraged me and provided unconditional support throughout my education.

CONTRIBUTORS AND FUNDING SOURCES

This work was supported by a thesis committee consisting of Professor Raquel Sitcheran of the Department of Molecular and Cellular Medicine, Professor Vishal Gohil of the Department of Biochemistry and Biophysics, Professor Farida Sorhabji of the Department of Neuroscience and Experimental Therapeutics, and Professor Weston Porter of the Department of Veterinary Integrative Biosciences.

Some analyses described in Chapter III were conducted by Linda Herrera de Lechuga, and cell lines were generated by Dong Lee of the Department of Molecular and Cellular Medicine.

This work was funded by a grant from the NIH (R01NS082554).

NOMENCLATURE

BM	Basement Membrane
BR3	BAFF receptor
CD30L	CD30 ligand
CD40L	CD40 ligand
cIAP	Cellular inhibitor of apoptosis
COX-2	cyclooxygenase-2
DiO	3,3'-Dioctadecyloxacarbocyanine Perchlorate
Drp1	Dynamin-related protein 1
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
Fn14	Fibroblast growth factor-inducible 14
GBM	Glioblastoma multiforme
IKKα/β	IkappaB kinase α/β
IL-1R	Interleukin 1 receptor
IκB	IkappaB

LTβR	Lymphotoxin-β receptor
MEFs	Mouse embryonic fibroblasts
NF-κB	Nuclear Factor-kappaB
NIK	NF-κB inducing kinase
pNIK-RFP	RFP reporter under the NIK promoter
RHD	Rel homology domain
ROI	Regions of interest
TLR4	Toll-like receptor 4
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor-associated factor
TWEAK	Tumor necrosis factor-like weak inducer of apoptosis

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1 Glioblastoma

Approximately, 80,000 people will be diagnosed with a primary brain tumor this year, with 16,000 people losing the battle yearly [1]. To adequately depict the features of the tumor and how "undifferentiated" the cancerous cells appear a grade system was developed (I, II, III, and IV) [2]. Glioblastoma multiforme (Glioblastoma, GBMs) are high grade (IV) malignant type of brain tumor that form in the central nervous system (CNS) [2,3]. According to American Brain Tumor Association, patients with GBMs have a median survival of about 15 months and five-year survival of 10% [1].

Despite innovations in cancer treatments such as radiation, chemotherapy, and surgical techniques the prognosis of patients' diagnosis with GBMs have not drastically improved over the years. This poor prognosis is primarily due to the aggressive proliferation, migration, and invasion properties of GBMs making it nearly impossible to contain and eradicate all cancerous cells without irritable harm to the patient. Typically, there is no defined boundary between the tumor and healthy brain tissue due to cancer cell invasion and diffusion of single cells or clusters of cancerous cells into the surrounding tissue; resulting in an incomplete resection and consequently a high recurrence rate [4,5]. The underwhelming advancement in therapies to combat GBMs has resulted in the reexamination of the cellular and molecular hallmarks contributing to the malignancy of GBMs to improve future therapies. Therefore, a strong potential for therapeutics targeting genes that attenuate the invasive potential of GBMs could significantly improve the prognosis of patients.

1.2 NF-κB Pathway

The nuclear factor-kappa B (NF-κB) pathway is responsible for a range of immune responses including cell death and survival (Figure 1) [6]. The NF-κB family consist of five inducible transcription factors NF-κB1 (p105/p50), NF-κB2 (p100/p52), RelA (p65), RelB, and c-Rel [6]. The NF-κB transcription factors are found as inactive cytoplasmic complexes. The transcription factors are structurally related proteins containing a highly conserved Rel homology domain (RHD) that is crucial for dimerization, nuclear translocation and DNA binding [6,7]. Both NF-κB1 and NF-κB2 are the products of precursor proteins p105 and p100 respectively, through partial proteasomal degradation of the C-terminal regions [6,7,8]. While p105 proteolytic processing into p50 is constitutive, p100 proteolytic processing into p52 is tightly regulated. NF-κB activation occurs through two signaling pathways the canonical and non-canonical pathway [6,7].

The canonical NF-κB pathway is known for its role in innate immune response, cell survival and proliferation, and inflammatory response. The canonical NF-κB pathway is activated upon stimulation of tumor necrosis factor receptor (TNFR), interleukin 1 receptor (IL-1R), and Toll-like receptor 4 (TLR4) resulting in a rapid and transient response [6]. Upon stimulation of the receptor TGFβ-activated kinase 1 (TAK1/MAP3K7) activates the IκB Kinase (IKK) complex that consist of IKKα, IKKβ, and regulatory IKKγ [7]. The activated IKK complex phosphorylates IκBα or p105 triggering ubiquitylation and proteasomal degradation, releasing p50/RelA or p50/c-REL heterodimers for nuclear translocation and transcription [6,7].

Conversely, the non-canonical NF-κB pathway activation through lymphotoxin-β receptor(LTβR), CD30 ligand (CD30L), CD40 ligand (CD40L), B cell activating factor (BAFF)

receptor (BR3) and Fibroblast growth factor-inducible 14 (Fn14) is slow and persistent [8,9]. Under unstimulated conditions NF-κB inducing kinase (NIK/MAP3K14) is bound to a degradation complex consisting of TNF receptor associated factor 2 (TRAF2), TRAF3 and cellular inhibitor of apoptosis (cIAP1/2) and targeted for proteasomal degradation as a method of its regulation [8,10,11]. Upon TNF family cytokine stimulation of the non-canonical pathway NIK is released from the degradation complex and stabilized where it phosphorylates and activates IKKα [12]. Upon its activation, IKKα phosphorylates precursor p100 for processing into p52 where p52 forms a heterodimer with RelB before translocation to the nucleus for transcription of non-canonical NF-κB genes [11,12,13]. The non-canonical pathway plays a major role in immunology, primarily in adaptive immune response, the regulation of lymphoid organ development, B cell survival and maturation, and the differentiation of osteoclasts [8,10,11].

Over the last few decades, NF-κB has been identified for its role in cancerous cells and the tumor microenvironment, as such has been targeted for therapy development [14]. Though NF-κB is known for its role in immune response, NF-κB has been shown to promote tumorigenesis through prevention of apoptosis and stimulation of cell proliferation [14,15]. Most notably, in breast cancer NF-κB promotes invasion through the upregulation of metalloproteinases (MMPs) expression and stimulation of the epithelial-mesenchymal transition (EMT) [14,16]. Although the exact mechanism is still largely unknown, NF-κB contributes to cell adhesion remodeling, one of the key factors that contribute to invasion and metastasis in cancerous cells [14,15,16].

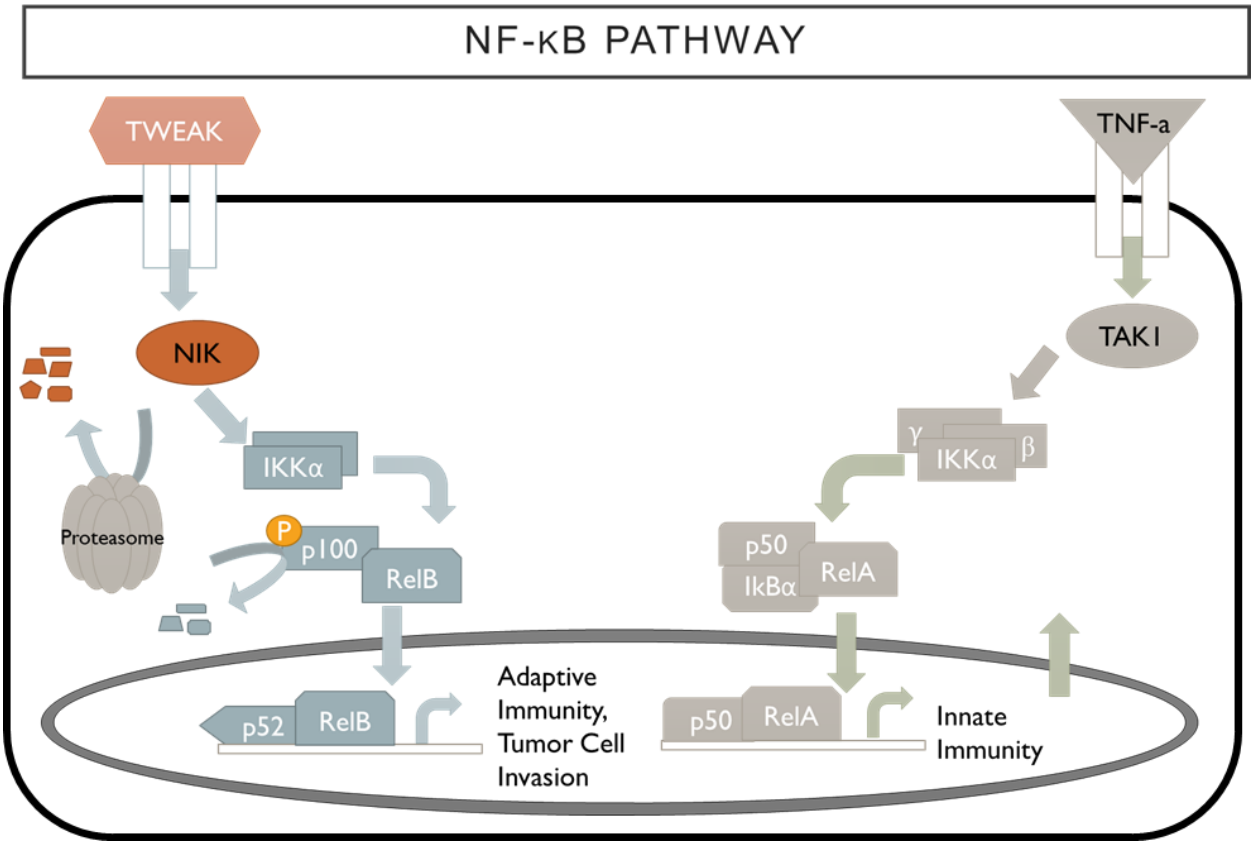

Figure 1: Two primary NF-κB pathways: canonical and non-canonical

The canonical NF-κB pathway under stimulated conditions (i.e. TNF-α), TAK1 phosphorylates an IKK complex. The IKK complex phosphorylates IκBα targeting the inhibitory protein for degradation triggering the release of RelA and p50 for nuclear translocation. The non-canonical NF-κB pathway under stimulated conditions (i.e. TWEAK) results in stabilization of NIK and phosphorylation of IKKα. Upon activation IKKα phosphorylates p100 for partial proteasomal degradation and release of p52 and RelB for nuclear translocation.

1.3 NF-κB Inducing Kinase and its regulation

NF-κB inducing kinase (NIK/MAP3K14), is a constitutively active serine/threonine kinase that is the primary driver of the non-canonical NF-κB pathway [17]. NIK consist of 947 amino acids with four primary domains: N-terminal domain (aa30-aa120 residues) in which TRAF3 binds, a negative regulatory domain (aa121-aa318 residues), a serine/threonine kinase domain (aa390-aa660 residues), and C-terminal domain where IKKα, and TRAF2 binds (aa660-aa947) [18]. NIK shares some homology to MAP3K, and upon examination of the homology it was determined that Thr 559 in the activation loop is required for kinase activity [18].

Under basal conditions newly synthesized NIK protein is bound to TRAF3 and targeted for ubiquitin dependent degradation [18,19]. TRAF3 recruits a degradation complex consisting of TRAF2, E3 ligases cIAP1 and cIAP2 for lysine (K) 48-linked ubiquitylation [13]. Upon ligand binding to the receptor activation of TRAF2 ligase activity occurs and K63-linked ubiquitination of cIAP occurs, resulting in K48 polyubiquitination of TRAF3 for degradation and NIK stabilization, accumulation and activation of the downstream non-canonical NF-κB pathway occurs [13].

NIK was first described as a NF-κB activating kinase, its role in B cell maturation and lymphoid organ development has been extensively examined [17,18]. Though recent studies have begun to delve into the role NIK plays in cancer [19,20,21]. Studies have shown that NIK expression is elevated in ovarian cancer, breast cancer, pancreatic cancer, leukemia, and melanoma [14,19,20]. NIK has been shown to mediate tumorigenesis through regulation of both survival and proliferation [14]. Though NIK expression is predominately thought to be regulated at the post translational level, the significance of the transcriptional regulation of NIK expression has yet to be illuminated.

1.4 Invasion

Although the hallmarks of cancer are constantly evolving as new insights into cancer develop activation of invasion and metastasis has remained an important corner stone [22]. Various contributing factors have been linked to invasion and metastasis including invadipodia formation, matrix metalloproteinase (MMP) expression, epithelial-mesenchymal transition, angiogenesis, mitochondrial trafficking to the leading edge, and leader invasion phenotype [22]. Invasion and metastasis are two of the leading contributing factors in mortality in patients diagnosed with cancer. The initial steps leading to metastasis of cancerous cells to distant organs can be broken down into these three steps: invasion, intravasation, and extravastion [23]. Through various pathway activations and obstructions cancerous cells adapt the ability to penetrate the surrounding tissue leaving the primary tumor site, initiating invasion [24]. In order for motile cells to invade through the basement membrane (BM) and extracellular matrix (ECM) substances are secreted to degrade the surrounding environment [23,24,25].

Various forms of invasion occur as cells begin to migrate away from the primary tumor and penetrate the surrounding environment [25]. There is not a single form of invasion, in fact invasion can be split into three categories: individual cell-migration, multicellular migration, and whole-tissue dynamics [23,24,25,26]. Individual cell migration encompasses single cell migration amoeboid and mesenchymal cell invasion with no cell-cell interactions as the cells invade [23,26]. Multicellular migration contains two types of invasion: multicellular streaming, and collective cell migration [23,24]. Multicellular streaming is comprised of cells that are invading as single cells with or without cell-cell junctions, that are following a pre-established path within the extracellular tissue structure or guided by a chemokine resulting in a directional invasion [23,24]. Conversely, collective invasion is a cohesive group of cells with cell-cell junctions maintained of prolong

intervals during the invasion process [23,24,26]. The main defining difference between multicellular streaming and collective invasion is the weak and transient cell-cell junctions observed in multicellular streaming as opposed to the maintained cell-cell junctions observed in collective invasion [24]. In collective invasion the leading edge maintains traction force, usually with the peripheral cells, although the cells inside the group may not contact the surrounding tissue at all during the invasion process [26,27]. A leader/follower invasion phenotype is often described when examining collective invasion. The "leader" cells are found at the tip of the leading edge with "follower' cells found trailing behind, to form collective cell invasion [27]. There are three well defined types of collective invasion: cluster (where the cells disassociate from the primary tumor and invade as a group of cells into the ECM), strand (can be as thin as one to two cells in diameter invading away from the primary tumor), or luminal structure (where space is generated within the strand of cells as it invades into the ECM) [23,24,25]. Finally, whole-tissue dynamics consist of tissue folding and expansive growth, both can lead to positional changes [23,24]. However, the cells are not actively invading into the surrounding tissue. It is important to note that transitions between the various types of invasion can occur and various types of invasion can be observed in a single tumor *in vivo* conditions providing insight into the plasticity of cancerous cells [25,26,27,28].

1.5. Thesis Goal

NF-κB plays a vital role in the lymphoid organ development, B cell maturation, and a host of both innate and adaptive immune responses [7,8]. Conversely, NF-κB has been linked to various disease formation and cancer progression through regulation of proliferation, survival and inflammatory signaling [7,11]. Upregulation of both the canonical and non-canonical NF-κB

pathways have been observed in various cancer types. However, significant emphasis is placed on the role the canonical NF-κB pathway plays on cancer, while the non-canonical pathway lacks the necessary illuminations.

Our lab has established a significant role for NIK as a crucial inducer of glioma invasion and tumor formation through alterations in cell size, shape and invadipodia formation [29,30]. All of which are key contributing factors to the initiation of invasion in cancer. Specifically, we have established two distinct pathways in which NIK drives invasion through the phosphorylation and activation of membrane type-1 matrix metalloproteinase (MT1-MMP/MMP14); and most recently its role in mitochondrial recruitment of dynamin-related protein 1 (Drp1) and mitochondrial dynamics [30,31,32].

Examination of NIK, the fundamental driver of the non-canonical NF-κB pathway, in cancer has shown significant elevated expression [14]. Yet, under basal conditions NIK protein is degraded as a mechanism for its regulation, therefore NIK protein levels in unstimulated conditions are extremely low and difficult to detect [14,15]. Regulation of NIK expression and activity is generally considered to be regulated at the post translational level. However, we have described a known activator of the non-canonical NF-κB pathway pro-invasive cytokine TNF-like Weak inducer of apoptosis (TWEAK) as an inducer of mRNA NIK expression [30]. Exploration into the induction of transcriptional NIK expression in a signal specific manor has not been well defined. Here, we examine whether the upregulation of NIK mRNA expression during invasion alters invasion potential to promote a leader cell phenotype.

CHAPTER 2

MATERIALS AND METHODS

2.1 Cell culture and reagents

BT25, BT114, and BT116 cell lines were obtained from human glioma patients as previously described [28]. These cell lines were maintained as spheroids in Neural Stem Cell (NSC) medium containing DMEM/F-12, 1X B-27 supplement minus Vitamin A, 1X Glutamax, 25 ng/ml EGF, 25 ng/ml bFGF and 1X Pen/Strep (Life Technologies). MEFs were cultured in DMEM medium with 10 % FBS and 1X Pen/Strep. MDA- MB231 and Panc1 were from ATCC (Manassas, VA) and cultured in RPMI medium with 10% FBS and 1X Pen/Strep. All cells were cultured at 37 °C with 95 % humidity and 5 % CO_2 .

2.2 Reagents

The following primary antibodies were purchased for immunofluorescent staining: NIK (ab7204). Collagen type I (#354249) was purchased from Corning, Corning, NY, USA. RhTWEAK (#310-06) was obtained from PeproTech, Rocky Hill, NY, USA. DiD and DiO were purchased from Invitrogen. Mangiferin from Mangiferia Indica Leaves (M3547-100MG) was obtained from Sigma Aldrich.

2.3 Three-dimensional collagen invasion assay

Monolayer invasion assays were performed as previously described [28]. Briefly, collagen type I (Corning, NY) was diluted to 2 mg/ml in DMEM/F-12 medium (1X Pen/Strep) and matrices polymerized in 96-well plates. 4 x 10 5 cells cultured in NSC or NSC+10 % serum were seeded in

triplicate in 100µl DMEM/F-12 (1x Pen/Strep, 1X Glutamax) without growth factors or serum. Cells were fixed with 3 % glutaraldehyde solution after 48 hours of invasion and stained with 0.1 % toluidine blue. Invasion density was quantified by counting cells below the plane of the monolayer by bright-field light microscopy using a 10 X 10 ocular grid at 10X or 20X magnification corresponding to a 1 mm 2 field. Numbers in at least three equivalent, random fields were counted (n = 3 wells each) and normalized to the corresponding control. All experiments were performed at least three times.

Live cell invasion assays were performed using glioma cells. The cells were collected and centrifuged at 1.0 rcf for 2.5 minutes, media was removed and disassociated with accutase for 9 minutes at room temperature before centrifugation at 1.0 rcf for 2.5 minutes. Accutase was removed and cells were resuspended in NSC media, then quantified. Approximately $2.0x10^6$ cells were transferred into a 15 mL conical tube and media was added to 2 mL with 12 uL of DiO added. Cells were incubated for 30 minutes at 37°C with DiO, followed by centrifugation at 1.0 rcf for 2 minutes. Cells were washed three times in NSC media before re-quantification. Cells were either used for monolayer invasion assay at 40,000 cells/well as previously described [25] or $1.2x10^6$ cells were incubated for one week at 37°C in NSC media before being embedded into 2.0 mg/ml collagen matrix (Figure 2).

After spheroid formation, spheres were collected at 1.0 rcf for 2.0 minutes and resuspended in 2 mL of fresh media. Collagen matrix was prepared and approximately 60 µL of resuspended spheres were added to matrix and 18 µL spheres embedded in collagen added to each well. Collagen was allowed to solidify for 2 hours at 37°C, before taking initial images. Images were acquired over a 72 hours time course.

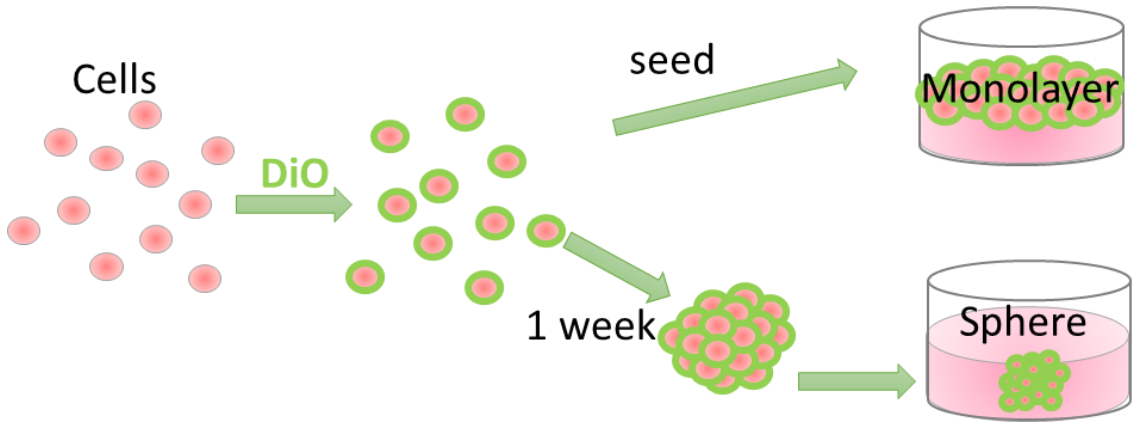

Figure 2: Schematic diagram of monolayer and sphere invasion protocol

Glioma cells were dissociated, followed by labeling with DiO for 30 minutes at 37° C. Cells were then washed in media and collected three times. Once washed the cells were either allowed to form a spheroid for 1 week or seed at 40,000 cells/well density for invasion. After one week spheroids were centrifuged at 1.0 rcf for 2 minutes, then resuspended in 2 mL. Approximately 60 uL of resuspended spheroids were added to make up to 1 mL of 2.0 mg/ml collagen matrix. Collagen matrix was allowed 2 hours to solidify at 37°C before initial confocal images were acquired. Z stack images were taken every at 3 to 4 hours for a 48 to 72 hours time period for both spheroid assay and monolayer invasion assay. Images were acquired using 0.4700 µm steps.

2.4 Immunofluorescence staining

Collagen embedded spheroids were seeded on an eight-well chamber slides (#80827, Ibidi, Munich, Germany) or 96 half-area well plate and allowed to adhere for 2 hours. During spheroid and monolayer invasion live cell imaging, cells were labeled with DiO or DiD for 30 minutes at 37°C before washing three times in media. Cells were allowed to invade for 48 to 72 hours then fixed with 4% paraformaldehyde, and permeabilized for 20 minutes with 0.3% Triton X-100 in

PBS. Cells were incubated overnight in 0.1% Triton X-100, 1% BSA in PBS at 4°C. The following day, cells were then incubated in 1% BSA for 1 hour at room temperature. Cells were counter stained with the nuclear stain Hoechst (Invitrogen, 3342).

2.5 Image acquisition

Images were attained with a Nikon TI A1R inverted confocal microscope with CFI60 Plan Apochromat Lambda 10x objective lens. Images were acquired with the following scan parameters: a "frame" scan mode of 1024 x 1024 pixels with a 16 bit depth and a grating of 3 rotations. Three-dimensional projections were obtained through Z stack images with a 0.4700 µm between each image.

2.6 RNA isolation, cDNA synthesis, and quantitative RT-qPCR

Total RNA was isolated from cells by Purelink™ RNA Mini Kit (Life Technologies). CDNA was synthesized from 1 µg total RNA using iScript reverse transcription supermix (Bio-Rad, Hercules, CA) following the manufacturer's protocol. Quantitative RT-PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA). The following primers were used: human NIK, 5'-CTAGTGCATGCTCTGCAAGG-3' and 5'-TGAGTTTCTCAGTGAGCAGGA-3'; mouse NIK, 5'-CCAGAATCGTCCCTCTCTAT-3' and 5'-GACAGCCCATTTGCTTTATG-3'; human MAP3K1, 5'-GGAGACAGCCCAGACAATAAA'-3' and 5'-ACCCGGAGCATCACAAATAG-3'; human MAP3K2, 5'-CTGAGACCAGCAAGGAAGTAAA-3' and 5'-CTGAGACCAGCAAGGAAGTAAA-3'; human MAP3K3, 5'-TCGTGCAGCCATGGTTATT-3' and 5'-TCGTGCAGCCATGGTTATT-

3’; human MAP3K4, 5’-ATCAGCAGTGCCCATGATAC-3’ and 5’-TGACGACTCAGGGACCTAAA-3’; human MAP3K5, 5’-CCCAGAGAGAGACAGCAGATA-3’, and 5’-CTCACTGAAAGAGCCCAGATAC-3’; human MAP3K6, 5’-CCAAAGAGCTCCGGCTAATA-3’ and 5’-CCAAAGAGCTCCGGCTAATA-3’; human MAP3K7, 5’-CGCCTGGTACAGGAACATAAA-3’ and 5’-TGCTGCTGACTTCTGATGAC-3’; human MAP3K8, 5’-GAGGTACCATGGTTGTCATCAG-3’ and 5’-CCTGTGGTCGTTGTCCATAAA-3’; human MAP3K9, 5’-CCGCCCATTCAGTTGTTAGA-3’ and 5’-CCTATCCAGAAAGCACGATAGAC-3’; human MAP3K10 5’-GGCACAAGACCACCAAGAT-3’ and 5’- GAGAAGAGGGAGAGACGGATAA-3’; human MAP3K11 5’- CCCTGAAGATCACCGACTTT-3’ and 5’- AAGGTGGAGGCCTTGATAAC-3’; human MAP3K12 5’- GCGCCACATAATCAACAGAAAG-3’ and 5’-GCCAGTGTCCCTAGAGTTTATC-3’; human MAP3K13 5’-CAGCTAGAAATGCGGGAGAA-3’ and 5’- TGGCATTGGGATGGATGATAG-3’; human PI3K 5’- GGGCTTTCTGTCTCCTCTAAAC-3’ and 5’- ATGTCTGGGTTCTCCCAATTC-3’; human AKT1 5’- CTACAACCAGGACCATGAGAAG-3’ and 5’-TCTTGAGCAGCCCTGAAAG-3’; human S6K 5’- CAAGGTGAGGGAGATAGGGATA-3’ and 5’-AAGGAAGGTAGACAGCAGAAAC-3’; human IKKα 5’-GTCAGGGAGACTTGATGGAATC-3’ and 5’- CATCTCTGTGCTGTCACTGTAG-3’; human IKKβ 5’- TCTCCTGCTGATTGTGTGTG-3’ and 5’- CTTGCCTTTCGGGTGTTATTTC-3’; human IKKγ 5’-TCACCAGCTCTTCCAAGAATAC-3’ and 5’-CTGGAGCTGCTGTTTGAGAT-3’; human IKKε 5’-TCCCACTCCCTCTGGTTTAT-3’ and 5’-AGACTGTGATGAGGTCGTTTG-3’; human TBK1 5’-GAAGGGCCTCGTAGGAATAAAG-3’ and 5’-CCCGAGAAAGACTGCAAGAA-3’; human PIK3R1 5’-

GCTTTGCCGAGCCCTATAA-3’ and 5’-ACATTGAGGGAGTCGTTGTG-3’; human GAPDH, 5’-GAAGGTGAAGGTCGGAGTC-3’ and 5’-GAAGATGGTGATGGGATTTC-3’. Expression of mRNA was normalized to either GAPDH expression levels. All experiments were performed at least three times with three replicates per sample.

CHAPTER 3

SPECIFIC mRNA INDUCTION OF NF-κB INDUCING KINASE (NIK) INCREASES INVASION POTENTIAL

3.1 Summary

Invasion and metastasis are one of the primary hallmarks of cancer [22]. Our lab has described an essential role for the non-canonical NF-κB pathway, specifically the vital role NIK plays in both tumor formation, and invasion [30]. Previously, we briefly described TWEAK, a known activator of the non-canonical NF-κB pathway, as an inducer of NIK mRNA expression [30]. Here we examine the role of NIK mRNA induction during invasion and alterations in invasion potential. We describe specific mRNA upregulation of NIK upon TWEAK stimulation, while no significant induction is observed upon TNF-α treatment. Interestingly, upon examination of cells actively undergoing invasion specific upregulation of NIK mRNA expression was observed. We establish that upon abolishment of downstream NF-κB mediators, NIK independently alters invasion potential. Upon TWEAK stimulation and NIK upregulation invasion characteristics shift from primarily individual cell invasion to a more collective multicellular invasion phenotype with high NIK expressing cells found at the leading edge. Taken together, these findings suggest that induction of NIK expression alters the invasion potential in glioma cells.

3.2 Introduction

NIK, the principle driver of the non-canonical NF-κB pathway, has been shown to contribute to tumorigenesis in various cancer types including breast, ovarian, and melanoma

cancer [5,9,16,17,18]. Our lab has shown a crucial role for NIK in glioma cell invasion, proliferation and tumor formation [30,31,32]. Specifically, the induction of NIK expression has drastic and potentially dire effects on tumor pathogenesis in various cancers. While the loss of NIK expression attenuates the observed aggressive malignant phenotype previously described. In glioma NIK dependent MT1-MMP expression has been observed, recent studies suggest that the induction and activation of MT1-MMP leads to a leader invasion phenotype through the modifications in the tumor microenvironment and paving the way for other cells to follow [28,29,33]. Additionally, NIK expression promotes glioma invasion through alterations in cell size and invadopodia formation [31]. The leading edge and invadopdia formation in the cell require excess energy for cytoskeleton and focal adhesion remodeling. Studies have suggested that changes in mitochondrial dynamics promote invasion and migration in cancer through the recruitment of fission mitochondria to the leading edge or invadopodia fostering invasion [34,35].

NIK, a constitutively active serine/threonine kinase, is generally considered to be regulated at the post-translational level through poly-ubiquitinoylation by a regulatory TRAF-cIAP complex. In basal conditions, NIK is constantly being turnover resulting in very low protein expression. Upon stimulation NIK is released from the regulatory complex, stabilized where it can then accumulate and further activate the downstream non-canonical NF-κB pathway promoting invasion [35,37]. Although, NIK regulation at the protein level has been examined greatly, there has not been extensive focus on induction of NIK mRNA expression. Here, I examine signal specific NIK mRNA induction and the role it plays during invasion.

3.3 Results

3.3.1 Stimulation of the non-canonical NF-κB pathway increases invasion

Previously, our lab defined a key role for the non-canonical NF-κB pathway in glioma invasion [36]. Specifically, NIK's role in tumor formation, invasion, invadiopodia formation, and MT1-MMP expression [30,31]. Upregulation of mRNA NIK expression was observed using qPCR analysis in wildtype MEFs, upon TWEAK stimulation (Figure 3A). However, there is no significant changes in NIK expression during TNF-α treatment. Additionally, stimulation with TWEAK in wildtype MEFs significantly increases invasion, while TNF-α treatment does not alter invasion potential (Figure 3B). Stimulation with TWEAK specifically induces NIK mRNA expression despite loss of NF-κB activity, as well as increases invasion potential. However, activation of the canonical NF-κB pathway upon TNF-α treatment shows no specificity in activation or stimulation of NIK or invasion in MEFs.

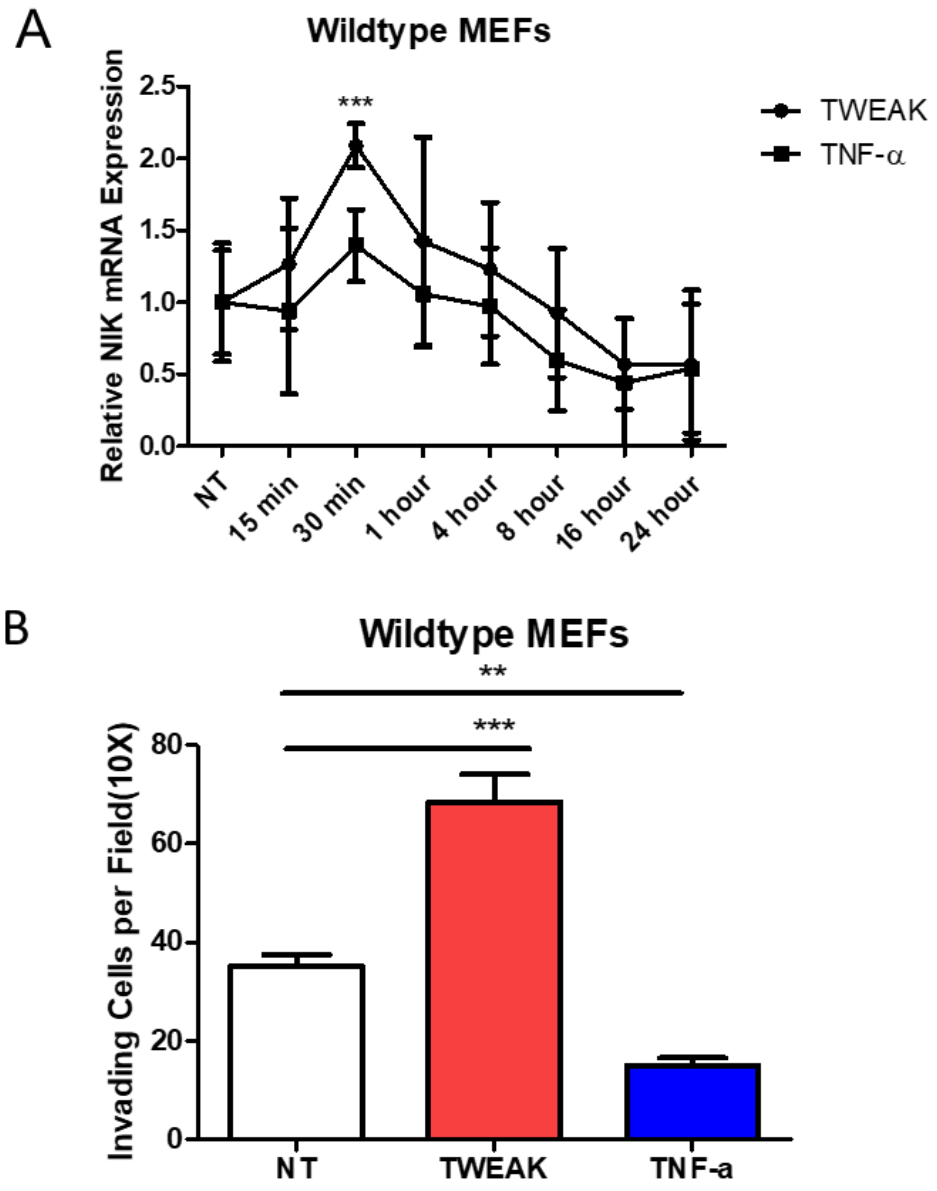

Figure 3: TWEAK specific induction of NIK mRNA expression promotes invasion.

NIK expression analysis was conducted over a 24 hour time course in A) Wildtype MEFs treated with either 10 ng/ml TWEAK or 10 ng/ml TNF-α. B) Three-dimensional collagen invasion assay was conducted under basal, 10ng/ml TWEAK, and 10ng/ml TNF-α stimulation for 48 hours. Invasion assays were conducted with an $n \geq 6$. One-Way ANOVA $p < 0.001$ was obtained comparing time points to NT condition.

3.3.2 Signal specific mRNA induction of NIK increases invasion potential in glioma

Despite TNF-like Weak inducer of apoptosis (TWEAK) being an established non-canonical NF-κB activator, its role in NIK specific mRNA induction has not been well defined [37]. Although NIK expression and activity are generally considered to be regulated at the post-translational level, our data demonstrates that NIK mRNA dramatically increases in response to treatment with pro-invasive cytokines (e.g. TWEAK). To further examine the effect on TWEAK stimulated induction of transcriptional NIK (MAP3K14) expression BT114 glioma cells were used for qPCR analysis. In conjunction with previously published data TWEAK results in specific induction of NIK at the mRNA level (Figure 4A). While stimulation with TNFα and canonical NF-κB activation does not increase NIK mRNA expression (Figure 4B). The transcriptional expression of other MAP3Ks and NF-κB genes were analyzed to examine specificity of transcriptional stimulated upregulation. Apart from NIK expression, MAP3K8 transcription levels were also elevated upon TWEAK treatment, although the upregulation of MAK3K8 expression was also observed during TNFα treatment. Suggesting the transcriptional response of MAP3K8 expression is potentially nonspecific. Upon further examination of the role NIK plays in invasion, data depicts an active induction of mRNA NIK expression during invasion (Figure 4C). Quantification of the invasion potential further suggest that the specific activation and upregulation of transcriptional NIK promotes invasion. Taken together with the transcriptional qPCR analysis of NIK, reporter cell lines were established with an RFP under the NIK promoter (pNIK-RFP) for live cell imaging and analysis. Live cell invasion three-dimensional projections show relative NIK expression, represented by RFP reporter, is induced in BT116 pNIK-RFP cells actively invading in a three-dimensional collagen matrix (Figure 5A). Measurement of NIK reporter RFP ROI shows a positive correlation of invasion distance and ROI intensity, suggesting that cells invading the

greatest distance have the highest NIK expression (Figure 5B). Transcriptional upregulation of NIK expression plays an important role in promoting invasion in glioma cells.

To examine the effects of inhibition therapeutic targeting of NIK regulation established NF-κB inhibitors were tested for NIK transcriptional inhibition. Mangiferin, a naturally occurring glucosylxanthone that can be found in mangos, proved to effectively inhibit NIK transcriptional upregulation [38,39,40]. Mangiferin has been defined for its anti-inflammatory, anti-proliferative, and anti-viral properties [40,41]. Recently, it has been defined for its apoptotic effects in leukemia, and more recently as an inhibitor of the NF-κB pathway [41,42]. Due to its know anti-proliferative and apoptotic characteristics a proliferation assay was conducted to establish a non-toxic concentration. Mangiferin treatment showed no observed anti-proliferation effects at 100 ug/ml concentration during a four-day period (Figure 6A). It is important to note when examined at 200 ug/ml mangiferin significantly decreased the proliferation rate compared to basal conditions. Comparisons of invasion potential were obtained with BT116 pNIK-RFP cells using a 100ug/ml concentration of mangiferin, 10ng/ml TWEAK, and 10 ng/ml TNF- (Figure 6B). Treatment with mangiferin significantly decreased the invasion potential of BT116 pNIK-RFP cells in comparison to the no treatment (NT) conditions. Although as expected, there was a significant increase in invasion upon TWEAK treatment. Interestingly, the combination of mangiferin and TWEAK treatment mimicked the invasion potential seen in the NT conditions, where TWEAK treatment able to rescue the lost invasion potential. The BT116 pNIK-RFP cells were utilized for live cell imaging and labeled with DiO to examine the relative NIK expression during invasion under various treated conditions (Figure 7). As seen in the quantification of invasion, the mangiferin with TWEAK treatment condition mimicked the invasion of the NT. The addition of treatment with TWEAK appears to combat the effects of mangiferin, though it is unable to further promote

invasion as seen in the TWEAK stimulated condition. Transcriptional induction of NIK plays a crucial role in cancer cell invasion and inhibition of its induction could prove a key therapeutic target.

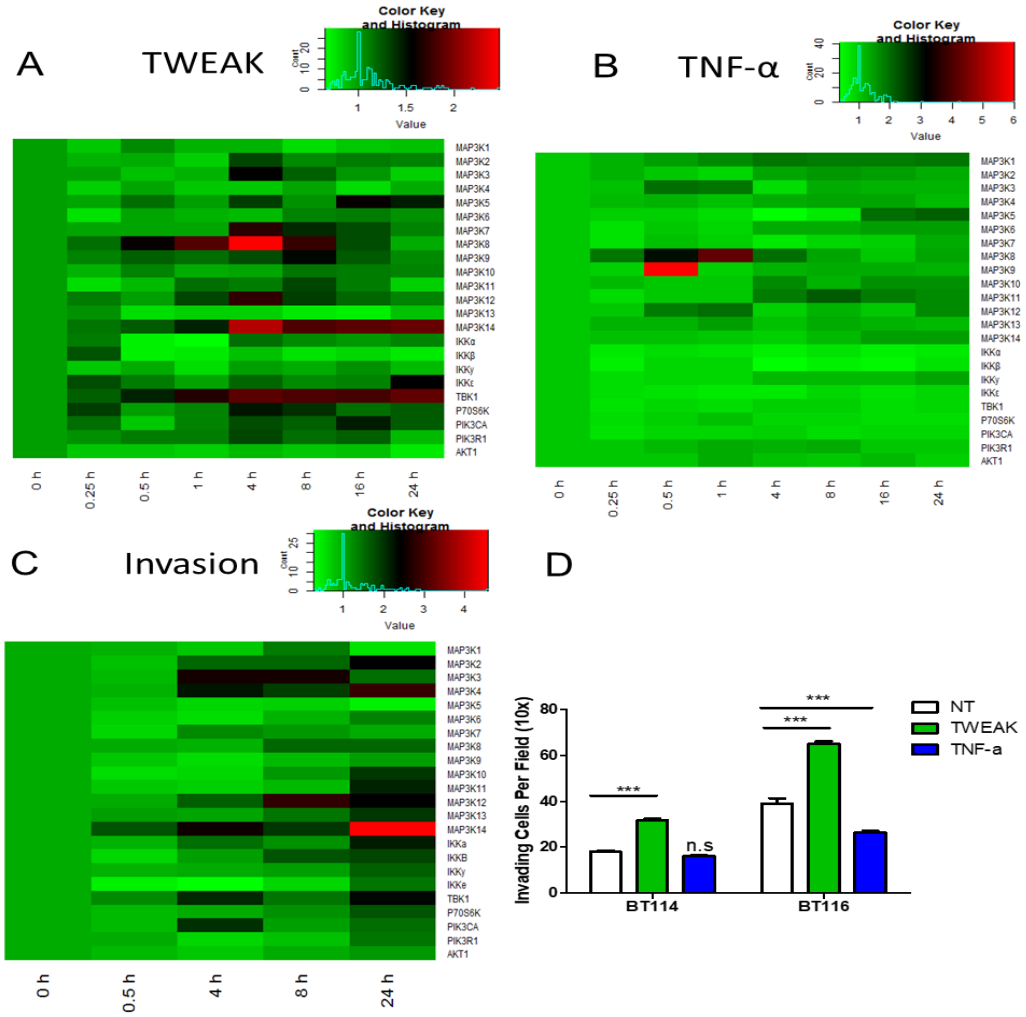

Figure 4: Signal specific NIK induction promotes invasion

RT-qPCR analysis was utilized to examine the expression of NF-κB proteins and MAP3Ks in BT114 glioma cells. Cells were treated with either A) TWEAK 10 ng/ml, B) TNF-α 10 ng/ml at 0, 0.25, 0.5, 1, 4, 8, 16, 24 hours. C) RT-qPCR analysis was utilized to examine the expression of NF-κB proteins and MAP3Ks in BT114 glioma cells (performed by Linda Herrera, PhD.). Cells were extracted from collagen matrix at 0, 0.5, 4, 8, 24 hours. Heatmap was generated using R studio heatmap2.0. D) Quantification of three-dimensional collagen invasion assay BT114 and BT116 glioma cells after 48 hours. Invasion was conducted under basal, TWEAK treatment (10ng/ml) and TNF- (10ng/ml). Statistical analysis was performed using Two-way ANOVA $p < 0.001$. Herrera, McFadden, Sitcheran unpublished.

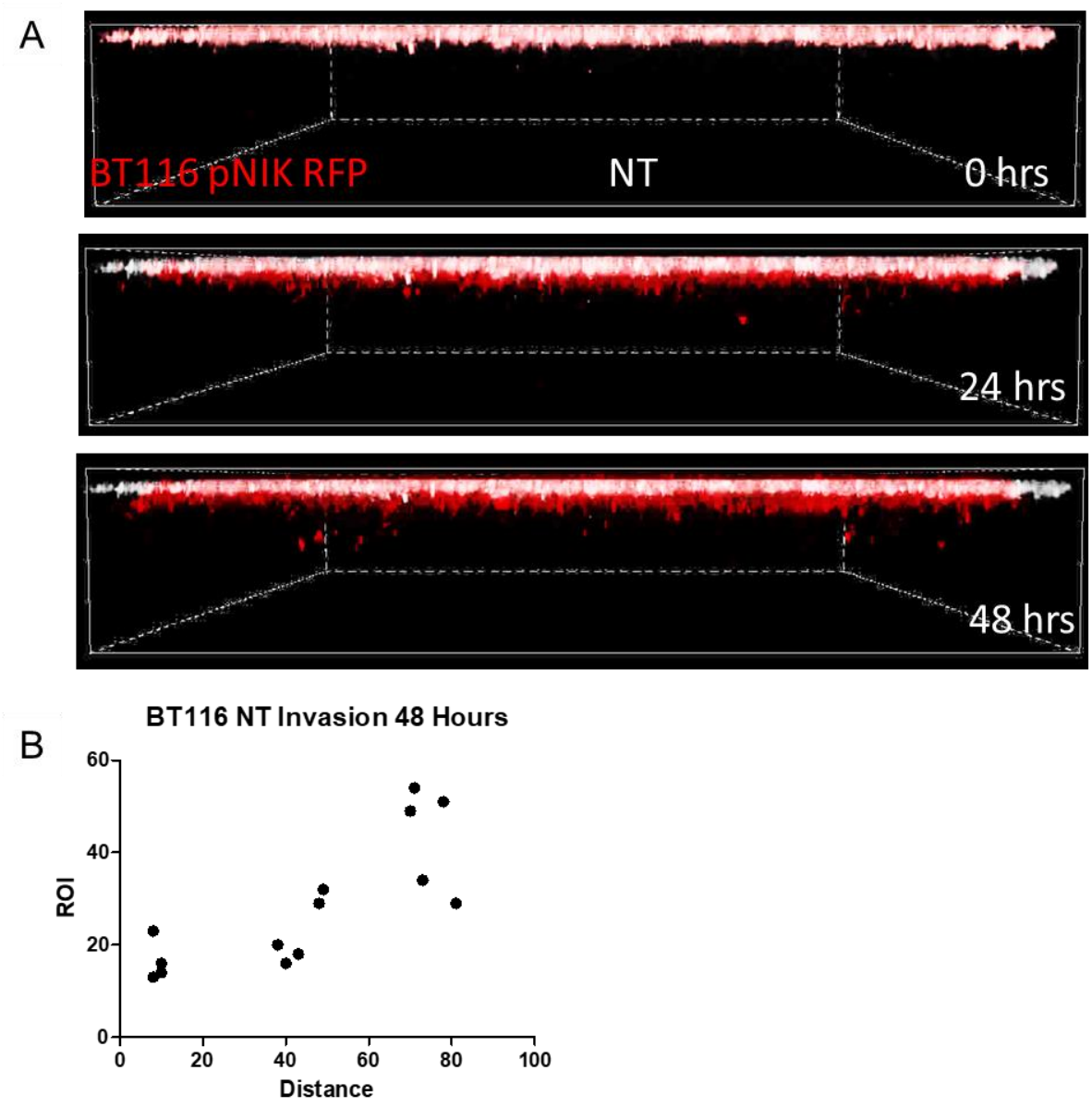

Figure 5: Invasion induces specific NIK mRNA expression

A) Live cell confocal microscopy was utilized to visualize RFP reporter under NIK promoter (pNIK-RFP) (Red) under unstimulated conditions (NT). Monolayer (0 hrs) was pseudo colored white and overlaid to reference invasion distance. B) Graph representation of RFP intensity after 48 hours and distance the cells invaded.

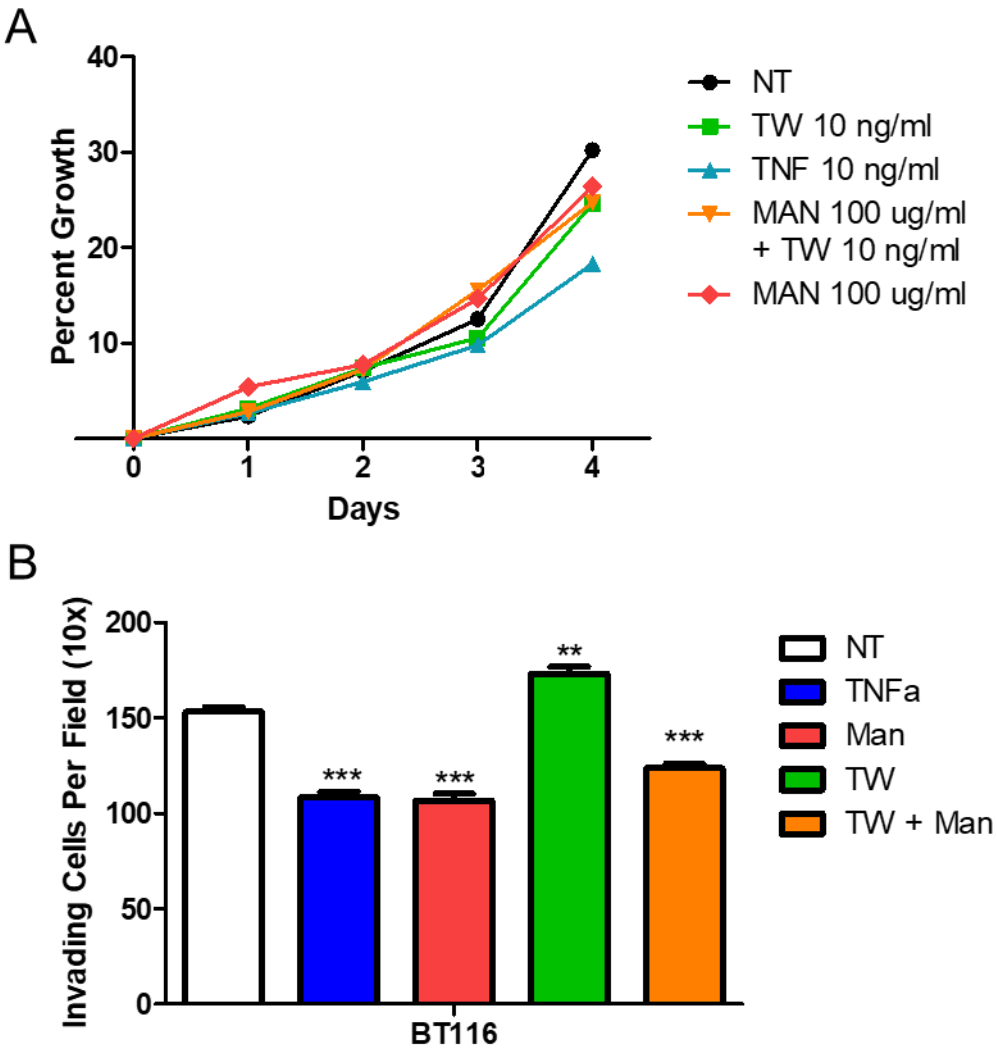

Figure 6: Mangiferin, a NIK inhibitor, attenuates TWEAK-induced glioma cell invasion

BT116 pNIK RFP cells were used for A) a proliferation assay was conducted to examine potential changes in proliferation after treatment stimulation. Cells were treated with or without TWEAK (TW) 10ng/ml, TNF-α 10ng/ml, or Mangiferin 100 ug/ml. B) BT116 pNIK RFP cells were treated with the aforementioned conditions for quantification of invasion. One-way ANOVA statistical analysis was performed compared to NT. $p < 0.001$

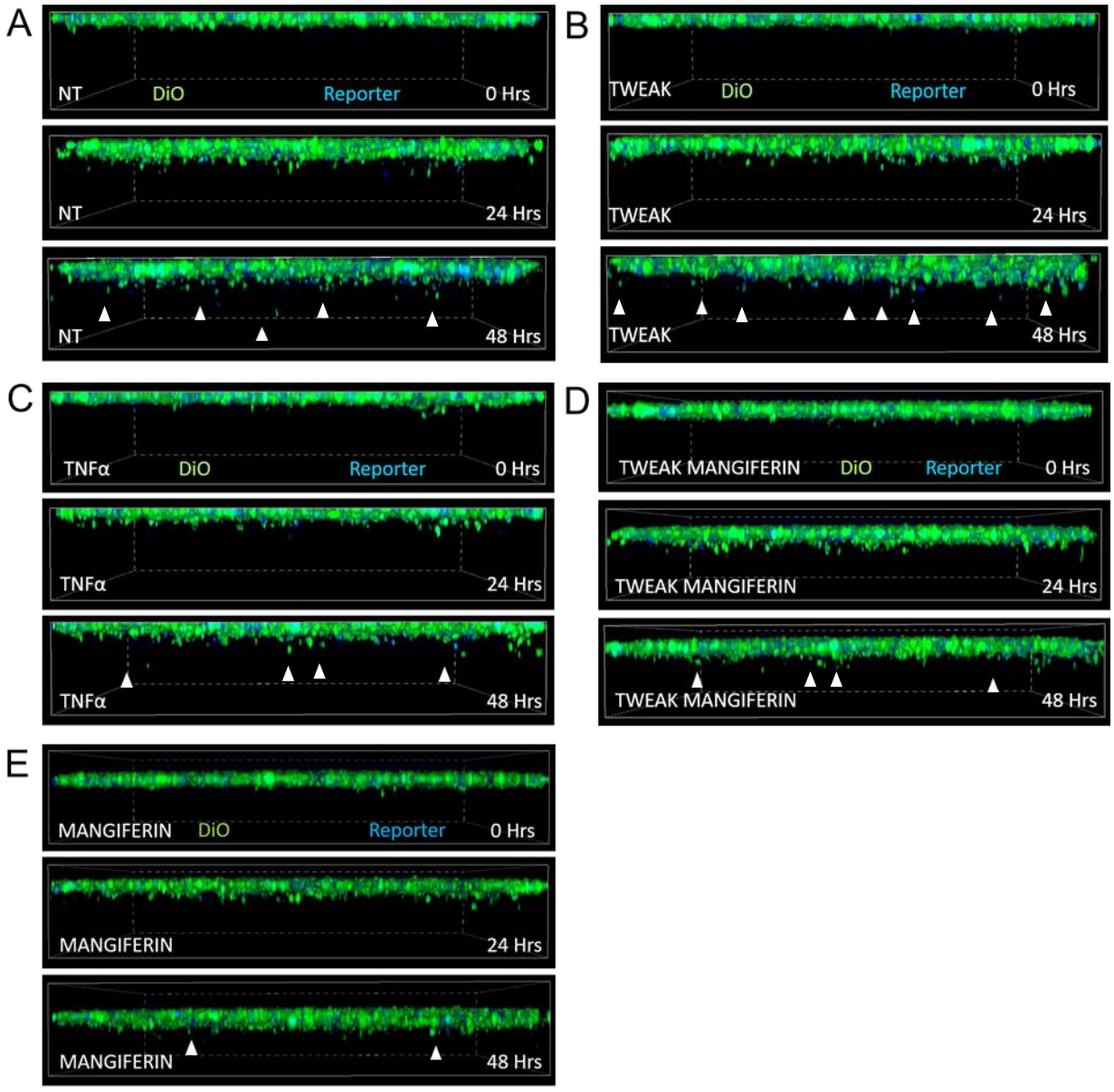

Figure 7: Alterations in NIK expression change invasion potential

BT116 pNIK RFP cells were labeled with DiO before being seeded on collagen matrix and allowed to invade for 48 hours. Live cell confocal microscopy was used to obtain Z-stack images over 48 hours with images acquired at 0 hours (0 Hrs), 24 hours (24 Hrs), and 48 hours (48 Hrs). Cells were treated with either A) NT, B) 10 ng/ml TWEAK, C) TNF-α, D) 10 ng/ml TWEAK with 100 ug/ml Mangiferin, and E) 100 ug/ml Mangiferin. White arrow heads indicate cells that have invaded into collagen matrix.

3.3.3 High NIK expressing cells induces invasion of follower cells through paracrine effects

As additional examinations of various cancers continue further illuminations into the heterogeneous makeup of tumors are revealing the complexity of a tumor population [22,29]. The invasion potential of cancerous cells appears to differ even within the same tumor which can result in a leader/follower invasion phenotype [27]. The leader/follower phenotype presents itself in two primary formats collective invasion where cell interaction is maintained as the cells invade or cells follow one specific cell but do not maintain cell-cell interactions [23,24,25]. Spheroid invasion assay show there is a positive correlation in invasion distance and relative NIK expression represented by RFP reporter in BT116 pNIK-RFP glioma cells (Figure 8, Figure 9). Examination of ROI categorized into leader, follower, and non-invading cells, show an increase in RFP signal in cells at the leading edge of the invading strands and cells in the periphery (Figure 9B). As cells invade into a 3D collagen matrix the higher the induction of RFP expression, where the cells with the highest expression are located on the periphery of the invading field (Figure 10). Interestingly upon TWEAK stimulation there is a shift in the invasion phenotype from individualized cell invasion to promoting a more collective invasion phenotype (Figure 11). To further understand the effects of high NIK expressing cells (BT25) on low NIK expressing cells (BT114) a cell mixing experiment was performed under both monolayer and spheroid invasion conditions. Notably, in the presence of high NIK expressing cells invasion was significantly increased, however there was a reduction in cell-cell collective invasion phenotype. Signifying that the presence of high NIK expressing cells increased the invasion potential of low NIK expressing cells, possibly by a secreted factor. To further examine the possibility of a secreted factor BT116 pNIK-RFP cells were treated with conditioned media taken from BT25 control, BT25 sgNIK, and BT25 NIK rescue glioma cells during invasion. There was a significant increase in invasion in cells exposed to BT25

control and BT25 NIK rescue conditioned media, while the BT25 sgNIK conditioned media had no effect on invasion compared to the unconditioned media (Figure 12). High NIK expressing cells induces invasion of less invasive cells through NIK dependent secreted factors, demonstrating that NIK promotes a leader cell phenotype.

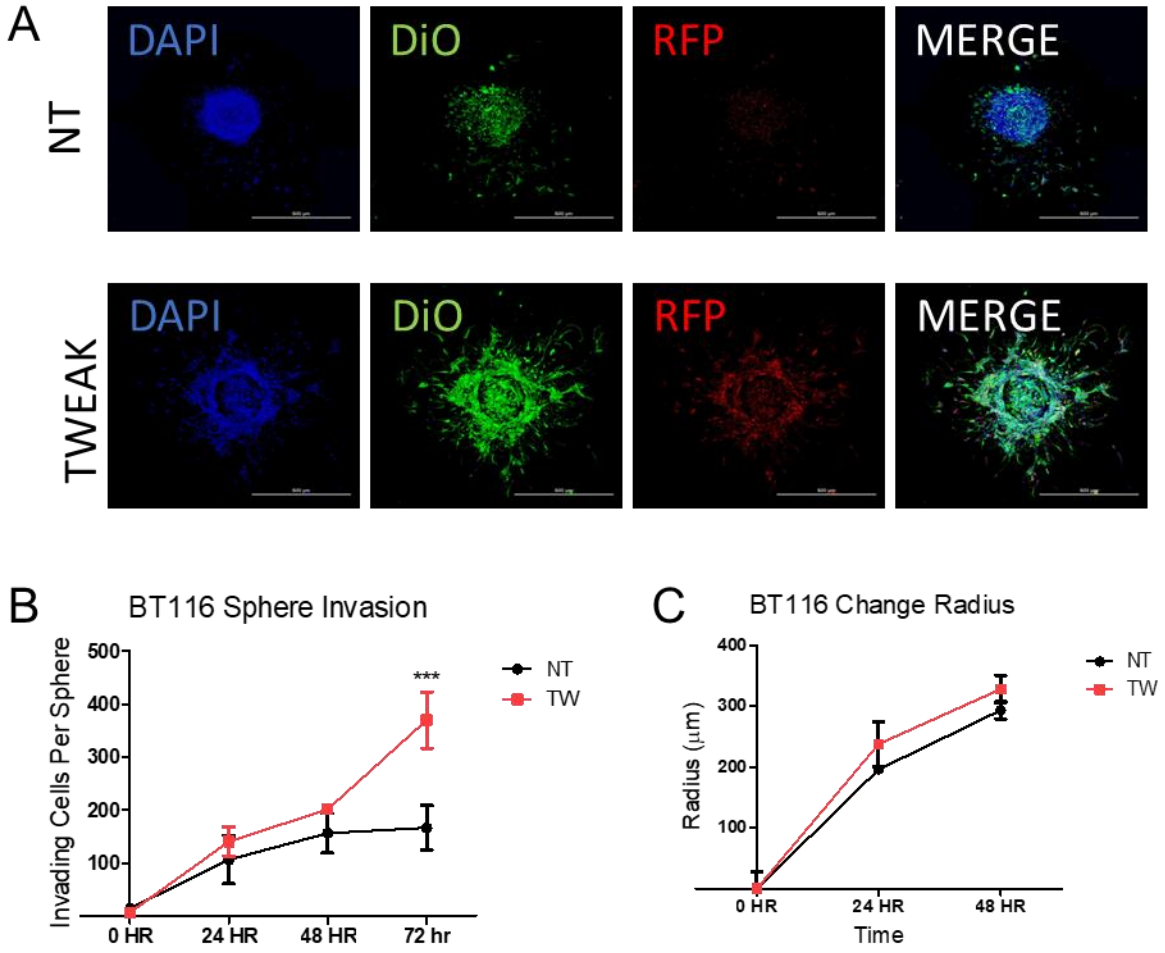

Figure 8: Upregulation of NIK expression induces collective invasion

A) BT116 pNIK RFP cells were used for a spheroid invasion assay. Spheroids were embedded in three-dimensional collagen matrix and allowed to invade for 72 hours. Confocal microscopy was used to image spheroids, labeled DiO (green), RFP reporter (red), and DAPI (blue). B) Image J particle analyze function was used to quantify the number of cells invading into the three-dimensional collagen matrix at various time points. C) Image J was used to measure the radius of the spheroids at various time points.

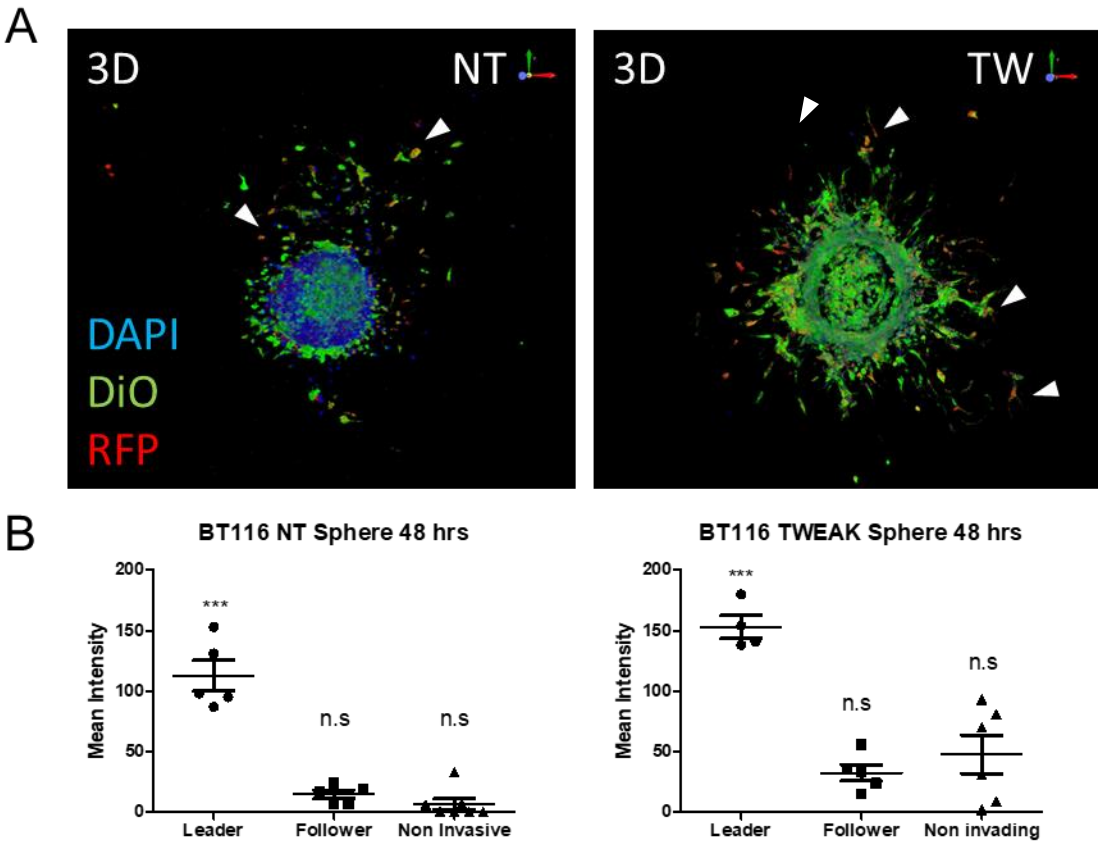

Figure 9: NIK expression correlates with invasion potential in glioma

A) Three-dimensional projection images were acquired using confocal microscopy Z-stack. BT116 pNIK-RFP cells were labeled with DiO (green), DAPI (blue), and NIK RFP promoter (red). Spheroids were either treated with or without TWEAK (TW) 10 ng/ml. C) RFP mean intensity was measured using ICY RIO program. Spheroid invasion assays were conducted in n≥3.

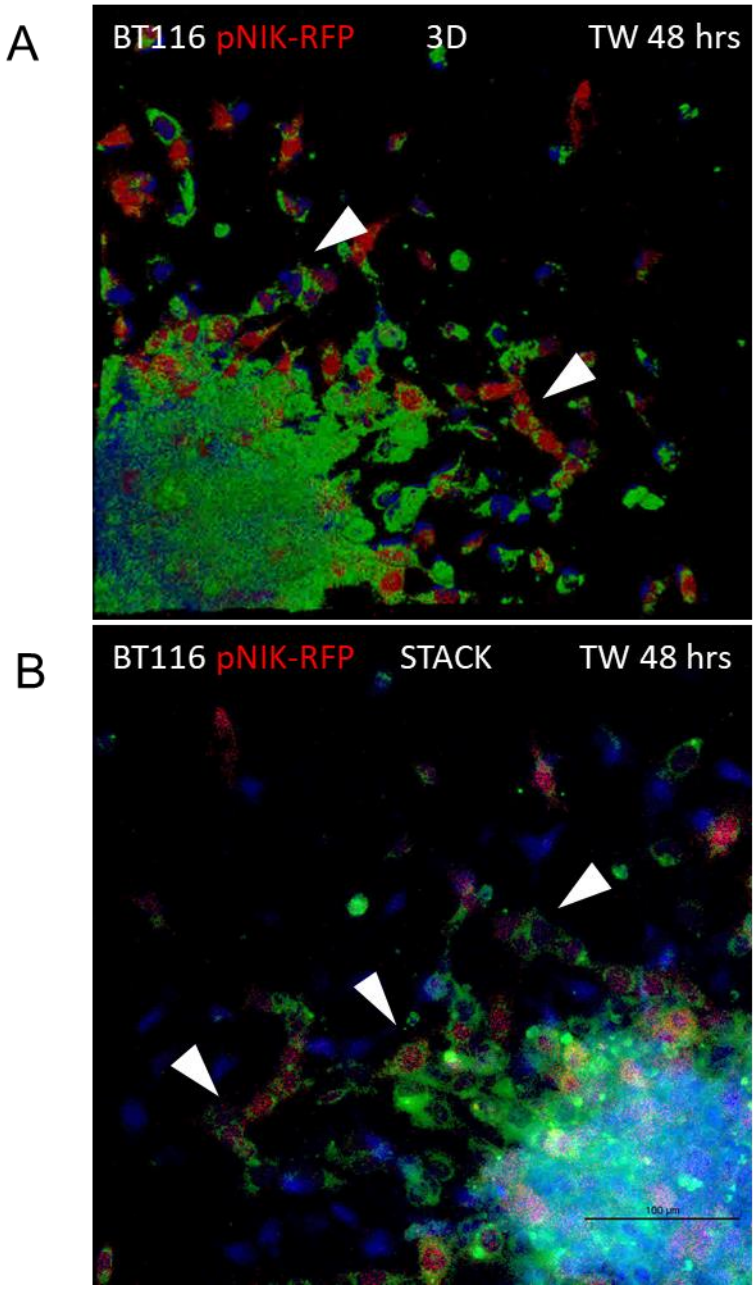

Figure 10: NIK expression levels are increased in collective invasion

BT116 pNIK RFP spheroids were embedded in three-dimensional collagen and treated with TWEAK (TW) for 48 hours. Cells were labeled with DiO (green), RFP reporter (red), and DAPI (blue). White arrow head point to areas of collective invasion and multicellular streaming. Represents A) a three-dimensional projection image, B) a single image from a Z stack of spheroids.

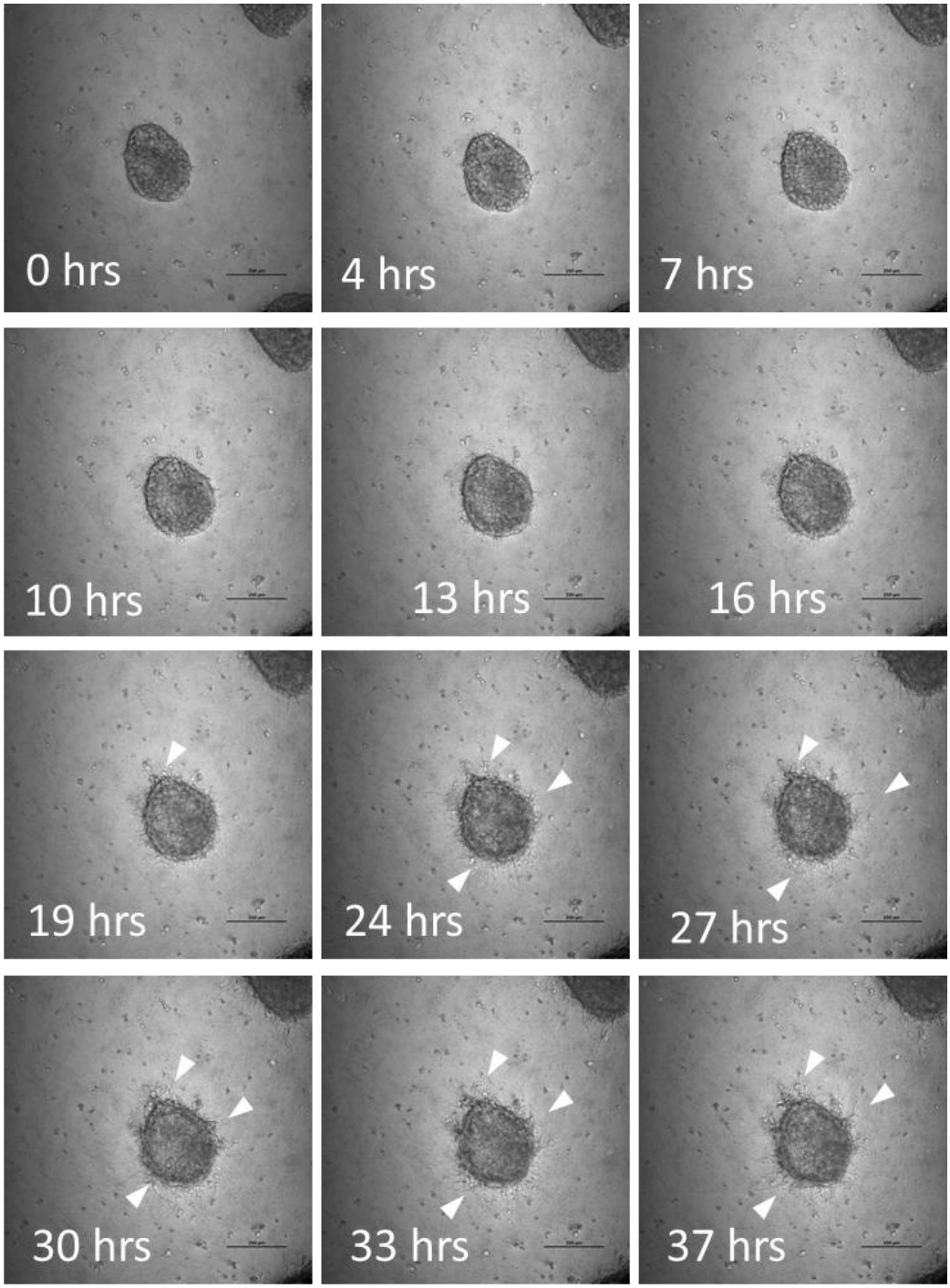

Figure 11: TWEAK stimulation induces collective invasion in glioma

Confocal microscopy live cell images were taken of BT116 glioma spheroids embedded in three-dimensional collagen over a 37 hours time course. Arrow heads indicate sprouting points of collective invasion.

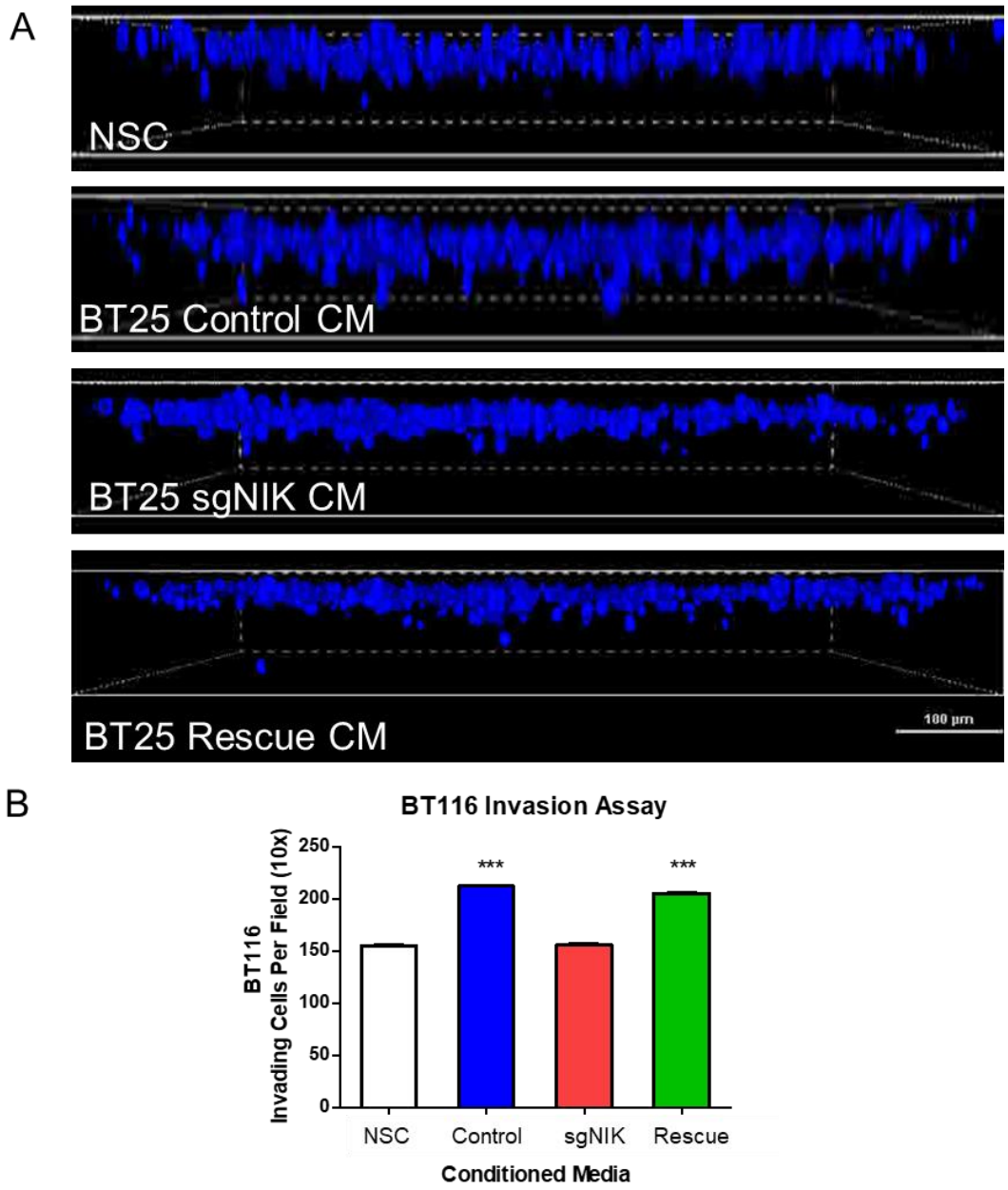

Figure 12: NIK dependent secreted factors alter invasion potential

A) BT116 glioma cells were stimulated with 25% conditioned media obtained from BT25 control, BT25 sgNIK, and BT25 sgNIK +mNIK (Rescue) cells were added to normal growth media (NSC). Cells were counter stained using Hoechst for visualization. B) Quantification of cells that were allowed to invade into a three-dimensional collagen matrix for 48 hours before quantification and analysis. One-way ANOVA statistical analysis was used to determine significance. $P \leq 0.0001$

CHAPTER 4

CONCLUSION

The hallmarks of cancer are constantly adapting as new insights are being provided, but invasion and metastasis have remained a constant area of interest [22]. While exploring the complexity of invasion and metastasis examination of contributing factors, gene expression and protein activation not only within the cell but the microenvironment must be take into consideration [28,43,44]. The tumorigenic role of the NF-κB pathway in cancer has been explored, with extensive focus on the canonical NF-κB pathway with little emphasis on the importance on the non-canonical NF-κB pathway [45,46,47]. This study explores the effects of transcriptional upregulation of NIK, the primary driver of the non-canonical NF-κB pathway, expression during invasion resulting in alterations of invasion potential of low invasive cells.

Previously, we described TWEAK an activator of non-canonical NF-κB as an inducer of transcriptional NIK expression [30]. Examination of NIK expression in MEFs showed signal specific induction upon TWEAK stimulation, with no significant induction of expression with TNF-α treatment (Figure 3). A further examination of the specificity of TWEAK induced NIK transcription in glioma showed MAP3K8 transcription was pointedly increased along with NIK (Figure 4A). While no response in NIK transcription by TNF-α stimulation was observed, though MAP3K8 was also unregulated suggesting that the response is not specific to TWEAK stimulation. Quantification of glioma invasion potential was examined after treatment with either TWEAK or TNF-α, with significant increase in invasion only observed in TWEAK stimulated conditions (Figure 3B). Therefore, the upregulation of MAP3K8 does not alter the invasion potential as there was no observed induction during TNF-α stimulated conditions. Interestingly, analysis during

invasion showed significant induction of NIK throughout the duration of invasion (Figure 9). Suggesting a specific active induction of mRNA NIK expression during invasion. Live cell imaging of RFP reporter for NIK expression in glioma cell lines showed an induction of RFP signal as the cells invading into the collagen matrix, with RFP signal increasing the further the cells invaded (Figure 9B). Providing a relative visualization of NIK expression in cells during invasion. To further examine the importance of NIK transcriptional upregulation during invasion glioma cells were treated with mangiferin, an established NF-κB inhibitor, to prevent NIK transcription (Figure 10). Treatment with low concentrations of mangiferin in combination with TWEAK, blocks the induction typically observed with TWEAK stimulation. Signifying the importance of NIK induction to promote invasion. Furthermore, treatment with mangiferin alone inhibits invasion and NIK expression. These findings suggest an essential role the transcriptional induction of NIK expression in promoting invasion.

To explore the heterogenous makeup of glioma a spheroid invasion assay was utilized to mimic tumor invasion [48]. Live cell imaging and quantification analysis using ImageJ showed a significant increase in BT116 pNIK-RFP cells invading into the collagen matrix in TWEAK treated when compared to untreated spheroids (Figure 8). Using RFP as a reporter for NIK to visualize relative NIK expression showed a distinct trend of high RFP cells at the periphery of the invasion field (Figure 8, 9, 10). Along with a notable shift in the invasion phenotype upon TWEAK stimulation from individual cell invasion and individual multicellular streaming to a more collective invasion phenotype. Examination of the ROI signal showed a significant increase in RFP signal in the cells located at the leading edge compared to the cells trailing behind or not invading. Signifying the induction of NIK expression during invasion promotes a leader cell phenotype resulting in a shift in the invasion potential of follower cells. Conditioned media derived

from NIK expressing cells induced invasion, while conditioned media from NIK knockout cells had no effect on invasion (Figure 12). Upregulation of NIK expression causes a shift in the invasion phenotype potentially through NIK dependent secreted factors. Previously, NIK has been described as activating PU.1 the transcription factor that regulates cyclooxygenase-2(COX-2) expression [43,44,45]. COX-2 is an inducible form of cyclooxygenase protein that converts arachidonic acid into prostaglandin, prostaglandin can be secreted from cells promoting cytoskeleton remodeling and promotion of invasion [43,44,45]. Perhaps the observed NIK dependent alteration in invasion potential is a result of upregulation of COX-2 expression in high NIK expressing cells.

This study has defined an essential role for transcriptional upregulation of NIK during invasion to promote an invasion leader phenotype. While the inhibition or abolishment of NIK induction attenuates glioma cell invasion. As well as describing a NIK dependent secreted factor that increases the invasion potential of follower cells through a shift to a collective invasion phenotype. A working model proposes the complexity of NIK expression and activity promoting tumorigenesis and invasion in glioma (Figure 17) [32]. Collectively, these findings establish NIK as a future potential target for cancer therapeutics.

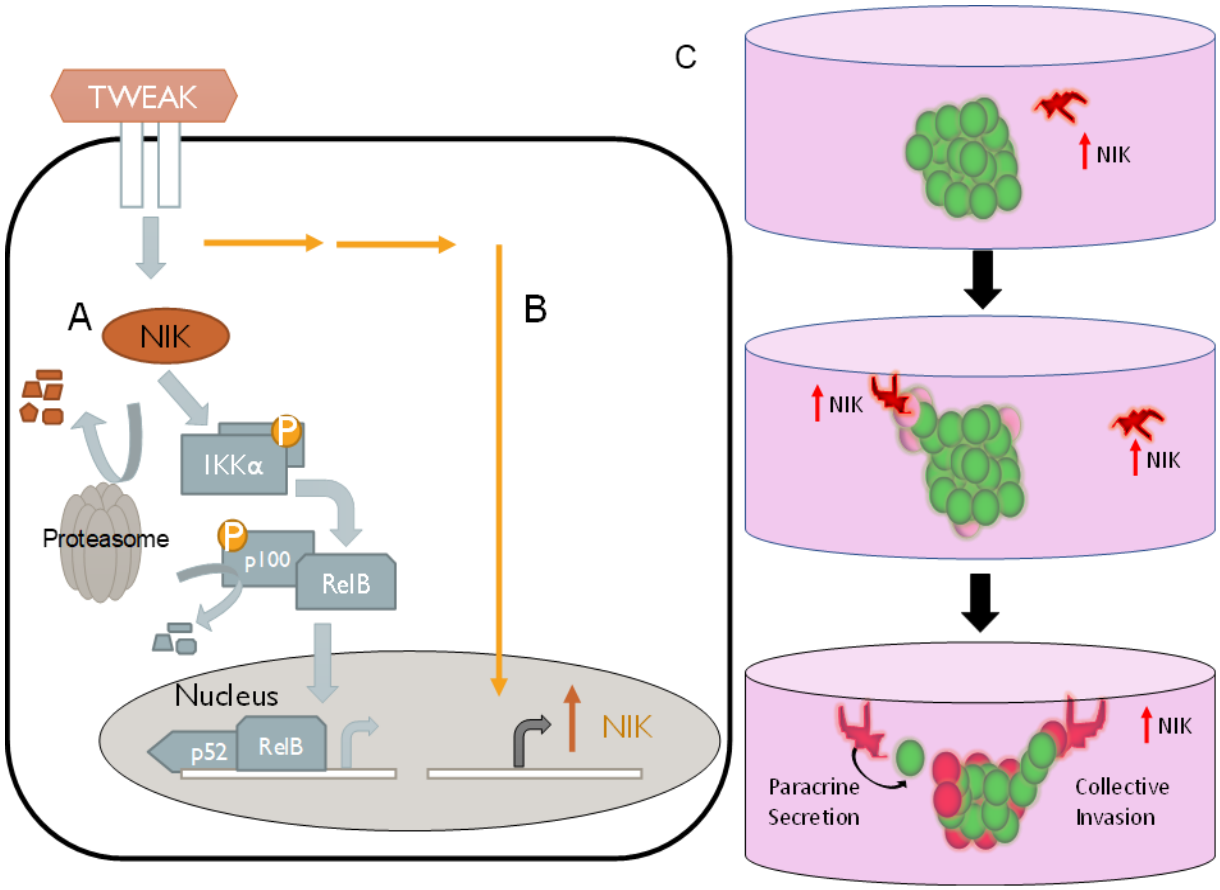

Figure 13: Model depicting promotion of invasion through specific induction of transcriptional NIK expression

Model shows two distinct pathways for NIK activity, along with an undefined pathway for induction of transcriptional NIK expression. A) NIK under basal conditions is constantly degraded as a mechanism for its regulation, upon stimulation NIK is stabilized and accumulates in the cytosol where NIK activates the downstream non-canonical NF-κB pathway. B) Upon TWEAK stimulation NIK transcriptional upregulation occurs, through as of yet unknown factors. C) Transcriptional upregulation of NIK expression promotes invasion through NIK dependent paracrine secreted factors, and collective invasion. Each of the described pathways contribute to the promotion of tumorigenesis, and invasion in glioma.

REFERENCES

1. American Brain Tumor Association. (n.d). Brain Tumor FAQs. Retreived from https://www.abta.org/about-brain-tumors/brain-tumor-faqs/
2. Hoelzinger, D.B., Demuth, T., Berens, M.E. (2007). Autocrine Factors That Sustain Glioma Invasion and Paracrine Biology in the Brain Microenvironment, *JNCI: Journal of the National Cancer Institute 99* (21), 1583–1593, https://doi.org/10.1093/jnci/djm187
3. Demuth T, Berens ME. (2004) Molecular mechanisms of glioma cell migration and invasion, *J Neurooncol , 70*, 217-28
4. Valastyan, S., and Weinberg, R.A. (2011). Tumor metastasis: molecular insights and evolving paradigms. Cell *147*, 275-292.
5. van Zijl, F., Krupitza, G., and Mikulits, W. (2011). Initial steps of metastasis: cell invasion and endothelial transmigration. Mutat Res *728*, 23-34.
6. Ramakrishnan, P., Wang, W., and Wallach, D. (2004). Receptor-specific signaling for both the alternative and the canonical NF-kappaB activation pathways by NF-kappaB-inducing kinase. *Immunity*. 21: 477–489
7. DiDonato JA, Mercurio F, Karin M. (2012) NF-kappaB and the link between inflammation and cancer. *Immunol Rev. 246*, 379–400. doi: 10.1111/j.1600-065X.2012.01099.x
8. Sun, S.-C. (2012). The noncanonical NF-κB pathway. *Immunological Reviews*, *246*(1), 125–140. http://doi.org/10.1111/j.1600-065X.2011.01088.x
9. Stephan, D., Sbai, O., Wen, J., Couraud, P.-O., Putterman, C., Khrestchatisky, M., & Desplat-Jégo, S. (2013). TWEAK/Fn14 pathway modulates properties of a human microvascular endothelial cell model of blood brain barrier. *Journal of Neuroinflammation*, *10*(1), 781. https://doi.org/10.1186/1742-2094-10-9
10. Razani, B., Reichardt, A.D., and Cheng, G. (2011). Non-canonical NF-κB signaling activation and regulation: principles and perspectives. *Immunol. Rev.* 244: 44–54
11. Thu, Y. M., & Richmond, A. (2010). NF-κB inducing kinase: A key regulator in the immune system and in cancer. *Cytokine and Growth Factor Reviews*, *21*(4), 213–226. https://doi.org/10.1016/j.cytogfr.2010.06.002
12. Chicheportiche, Y., Bourdon, P. R., Xu, H., Hsu, Y. M., Scott, H., Hession, C., Garcia, I., Browning, J. L. (1997). TWEAK, a new secreted ligand in the tumor necrosis factor family

that weakly induces apoptosis. *Journal of Biological Chemistry*, *272*(51), 32401–32410. https://doi.org/10.1074/jbc.272.51.32401

13. Brown, S. A. N., Cheng, E., Williams, M. S., & Winkles, J. A. (2013). TWEAK-Independent Fn14 Self-Association and NF-κB Activation Is Mediated by the C-Terminal Region of the Fn14 Cytoplasmic Domain. *PLoS ONE*, *8*(6). https://doi.org/10.1371/journal.pone.0065248
14. Xia, Y., Shen, S., & Verma, I. M. (2014). NF-κB, an active player in human cancers. *Cancer Immunology Research*, *2*(9), 823–830. http://doi.org/10.1158/2326-6066.CIR-14-0112
15. Schattner E.J., Furman R.R., Bernal A. (2006) NF-κB in Human Cancers. In: Liou HC. (eds) NF-κB/Rel Transcription Factor Family. *Molecular Biology Intelligence Unit*. Springer, Boston, MA
16. Chin-Chung, L., Choa-Lin, K., Yi-Ping, H., Cheng-Yen, C., Ming-Jie, H., Yung, L. C., Fu-Shin, C., Jing-Gung C. (2018). Demethoxycurcumin Suppresses Migration and Invasion of Human Cervical Cancer HeLa Cells via Inhibition of NF-κB Pathways. *Anticancer Res 38* (5), 2761-2769.
17. Sun, S.-C. (2010). Controlling the Fate of NIK: A Central Stage in Noncanonical NF-κB Signaling. *Science Signaling*, *3*(123), pe18. http://doi.org/10.1126/scisignal.3123pe18
18. Liu, J., Sudom, A., Min, X., Cao, Z., Gao, X., Ayres, M., … Wang, Z. (2012). Structure of the nuclear factor κB-inducing kinase (NIK) kinase domain reveals a constitutively active conformation. *Journal of Biological Chemistry*, *287*(33), 27326–27334. https://doi.org/10.1074/jbc.M112.366658
19. Annunziata, C.M., Davis, R.E., Demchenko, Y., Bellamy, W., Gabrea, A., Zhan, F., Lenz, G., Hanamura, I., Wright, G., Xiao, W., *et al.* (2007). Frequent engagement of the classical and alternative NF-kappaB pathways by diverse genetic abnormalities in multiple myeloma. Cancer Cell *12*, 115-130.
20. Demchenko, Y. N., Brents, L. a, Li, Z., Bergsagel, L. P., McGee, L. R., & Kuehl, M. W. (2014). Novel inhibitors are cytotoxic for myeloma cells with NFkB inducing kinase-dependent activation of NFkB. *Oncotarget*, *5*(12), 4554–66. https://doi.org/10.18632/oncotarget.2128

21. Uno M, Saitoh Y, Mochida K, Tsuruyama E, Kiyono T, et al. (2014) NF-kB Inducing Kinase, a Central Signaling Component of the Non-Canonical Pathway of NF-kB, Contributes to Ovarian Cancer Progression. *PLoS ONE, 9,* 2, e88347 1-12. doi:10.1371/journal.pone.0088347
22. Hanahan, D., Weinberg, R. A. (2011). Hallmarks of Cancer: The Next Generation. Cell, 144, 646-674. https://doi.org/10.1016/j.cell.2011.02.013
23. Friedl, P., Locker, J., Sahai, E., & Segall, J. E. (2012). Classifying collective cancer cell invasion. *14*(8), 777–784.
24. Ilina, O., & Friedl, P. (2009). Mechanisms of collective cell migration at a glance. *Journal of Cell Science*, *122*(18), 3203–3208. https://doi.org/10.1242/jcs.036525
25. Peter, F., Stephanie, A. (2011). Cancer Invasion and the Microenvironment: Plasticity and Reciprocity. *Cell 147*, 992-1009
26. Lintz, M., Munoz, A., Reinhart-King, C.A. (2017). The Mechanics of Single Cell and Collective Migration of Tumor Cells. *Journal of Biomechanical Engineering, 139*. 021005-1- 021005-9.
27. Yamaguchi, N., Mizutani, T., Kawabata, K., & Haga, H. (2015). Leader cells regulate collective cell migration via Rac activation in the downstream signaling of integrin β1 and PI3K. *Scientific Reports*, *5*. https://doi.org/10.1038/srep07656
28. Lorger, M. (2012). Tumor Microenvironment in the Brain. *Cancers, 4.* 218-243; doi:10.3390/cancers4010218
29. Hallou, A., Jennings, J., Kabla, A.J. (2017). Tumour heterogeneity promotes collective invasion and cancer metastatic dissemination. *R.Soc.opensci, .4*, 1-8.
30. Cherry, E. M., Lee, D. W., Jung, J.-U., & Sitcheran, R. (2015). Tumor necrosis factor-like weak inducer of apoptosis (TWEAK) promotes glioma cell invasion through induction of NF-κB-inducing kinase (NIK) and noncanonical NF-κB signaling. *Molecular Cancer*, *14*(1), 9. http://doi.org/10.1186/s12943-014-0273-1
31. Duran, C. L., Lee, D. W., Jung, J.-U., Ravi, S., Pogue, C. B., Toussaint, L. G., Bayless, K. J., Sitcheran, R. (2016). NIK regulates MT1-MMP activity and promotes glioma cell invasion independently of the canonical NF-κB pathway. *Oncogenesis*, *5*(6), e231–. http://doi.org/10.1038/oncsis.2016.39

32. Jung, J. U., Ravi, S., Lee, D. W., McFadden, K., Kamradt, M. L., Toussaint, L. G., & Sitcheran, R. (2016). NIK/MAP3K14 Regulates Mitochondrial Dynamics and Trafficking to Promote Cell Invasion. *Current Biology*, *26*(24), 3288–3302. https://doi.org/10.1016/j.cub.2016.10.009
33. Wang, D. J., Ratnam, N. M., Byrd, J. C., & Guttridge, D. C. (2014). NF-κB functions in tumor initiation by suppressing the surveillance of both innate and adaptive immune cells. *Cell Reports*, *9*(1), 90–103. https://doi.org/10.1016/j.celrep.2014.08.049
34. Zhao, J., Zhang, J., Yu, M., Xie, Y., Huang, Y., Wolff, D. W., Abell, Peter W., Tu, Y. (2013). Mitochondrial dynamics regulates migration and invasion of breast cancer cells. *Oncogene*, *32*(40), 4814–4824. http://doi.org/10.1038/onc.2012.494
35. Caino, M. C., & Altieri, D. C. (2015). Cancer cells exploit adaptive mitochondrial dynamics to increase tumor cell invasion. *Cell Cycle*, *14*(20), 3242–3247. http://doi.org/10.1080/15384101.2015.1084448
36. Raychaudhuri B, Han Y, Lu T, Vogelbaum MA. (2007). Aberrant constitutive activation of nuclear factor kappaB in glioblastoma multiforme drives invasive phenotype. *J Neurooncol. 85*, 39–47. doi: 10.1007/s11060-007-9390-7.
37. Lin, X., Mu, Y., Cunningham, E. T., Marcu, K. B., Geleziunas, R., & Greene, W. C. (1998). Molecular determinants of NF-kappaB-inducing kinase action. *Molecular and Cellular Biology*, *18*(10), 5899–5907. https://doi.org/10.1128/MCB.18.10.5899
38. Takeda, T., Tsubaki, M., Kino, T., Yamagishi, M., Iida, M., Itoh, T., … Nishida, S. (2016). Mangiferin induces apoptosis in multiple myeloma cell lines by suppressing the activation of nuclear factor kappa B-inducing kinase. *Chemico-Biological Interactions*, *251*, 26–33. https://doi.org/10.1016/j.cbi.2016.03.018
39. Li, H. H., Huang, J., Yang, B., Xiang, T., Yin, X., Peng, W., … Ren, G. (2013). Mangiferin exerts antitumor activity in breast cancer cells by regulating matrix metalloproteinases, epithelial to mesenchymal transition, and β-catenin signaling pathway. *Toxicology and Applied Pharmacology*, *272*(1), 180–90. https://doi.org/10.1016/j.taap.2013.05.011
40. Takeda, T., Tsubaki, M., Sakamoto, K., Ichimura, E., Enomoto, A., Suzuki, Y., … Nishida, S. (2016). Mangiferin, a novel nuclear factor kappa B-inducing kinase inhibitor, suppresses metastasis and tumor growth in a mouse metastatic melanoma model. *Toxicology and Applied Pharmacology*, *306*, 105–112. https://doi.org/10.1016/j.taap.2016.07.005

41. Gold-Smith, F., Fernandez, A., & Bishop, K. (2016, July 1). Mangiferin and cancer: Mechanisms of action. *Nutrients*. MDPI AG. https://doi.org/10.3390/nu8070396
42. Jeong, J. J., Jang, S. E., Hyam, S. R., Han, M. J., & Kim, D. H. (2014). Mangiferin ameliorates colitis by inhibiting IRAK1 phosphorylation in NF-κB and MAPK pathways. *European Journal of Pharmacology*, *740*, 652–661. https://doi.org/10.1016/j.ejphar.2014.06.013
43. Azim, A.C., Wang, X., Park, G.Y., Sadikot, R.T, Cao, H., Mathew, B., Atchison, M., van Breemen, R.B., Joo, M., Christman, J.W. (2007). NF-κB-Inducing Kinase Regulates Cyclooxygenase 2 Gene Expression in Macrophages by Phosphorylation of PU.1. *J Immunol*, *179*, 11, 7868-7875; DOI: https://doi.org/10.4049/jimmunol.179.11.7868
44. Shi, G., Li, D., Fu, J., Sun, Y., Li, Y., Qu, R., Jin, X., Li,. D. (2015). Upregulation of cyclooxygenase-2 is associated with activation of the alternative nuclear factor kappa B signaling pathway in colonic adenocarcinoma. *Am J Transl Res, 7*, 9, 1612-1620
45. Zhao, J., Zhu, J., Lv, X., Xing, J., Liu, S., Chen, C., & Xu, Y. (2017). Curcumin potentiates the potent antitumor activity of ACNU against glioblastoma by suppressing the PI3K/AKT and NF-kB/COX-2 signaling pathways. *OncoTargets and Therapy*, *10*, 5471–5482. https://doi.org/10.2147/OTT.S149708
46. Bhat KP, Balasubramaniyan V, Vaillant B, Ezhilarasan R, Hummelink K, Hollingsworth F, et al. (2013). Mesenchymal differentiation mediated by NF-kappaB promotes radiation resistance in glioblastoma. *Cancer Cell, 24*, 331–346. doi: 10.1016/j.ccr.2013.08.001.
47. Guan, F., Wang, L., Hao, S., Wu, Z., Bai, J., Kang, Z., Zhou Q., Chang, H., Yin, H., Li, D., Tian, K., Ma, J., Zhang, G., Zhang, J. (2017). Retinol dehydrogenase-10 promotes development and progression of human glioma via the TWEAK-NF-κB axis. *Oncotarget*, *8*(62), 105262–105275. https://doi.org/10.18632/oncotarget.22166
48. Calderwood, S.K. (2013). Tumor heterogeneity, clonal evolution, and therapy resistance: an opportunity for multitargeting therapy. Discov Med *15*, 188-194.