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ANALYSIS OF NFI-X3 AND STAT3 INTERACTION AND ITS FUNCTION

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science
at Virginia Commonwealth University.

by

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Vita

Etsegenet Tizazu was born in Gondar, Ethiopia on January 16, 1988. At the age of nine, she and her family moved to the United States. She received her Bachelor's degree in Brain, Behavior and Cognitive Sciences from the University of Michigan in 2008, at the age of 20. After graduating, she moved to Richmond, Virginia where she enrolled as a graduate student at Virginia Commonwealth University. In January of 2010, she enrolled in the Master's in Biochemistry program. On May 21, 2011, she graduated from Virginia Commonwealth University with a Master's in Molecular Biology and Biochemistry. Upon graduation, she will begin her medical education at Wayne State University School of Medicine. Upon completion of her medical degree, she hopes to travel to Ethiopia to open a clinic where she will provide free health care to the disabled and the less fortunate. Additionally, she hopes to educate Ethiopians about disability and mental health to help put an end to the stigma that exists there about these conditions.

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List of Abbreviations

AML-1	acute myeloid leukemia 1
Brg1	brahma-related gene 1
C/EBP	CCAAT-enhancer-binding proteins
CBP	CREB-binding protein
cDNA	complementary DNA
CIS	cytokine-inducible SH2 proteins
COX-2	cyclooxygenase-2
DMEM	Dulbecco's modification of Eagle's medium
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGFR	EGF receptor
ERK	extracellular signal-regulated kinase
FBS	fetal bovine serum
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GAS	gamma interferon activation site
GBM	glioblastoma multiforme

GFAP	glial fibrillary acidic protein
GLUT4	glucose transporter type 4
HEK	human embryonic kidney
IFN- $\alpha/\beta/\gamma$	interferon- $\alpha/\beta/\gamma$
IL	interleukin
IRF	IFN regulatory factor
ISRE	interferon-sensitive responsive element
JAK	Janus kinase
MAPK	mitogen-activated protein kinase
NFI	nuclear factor one
NF- κ B	nuclear factor kappa b
NLS	nuclear localization signal
OSM	oncostatin M
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PI3K	phosphoinositide 3-kinase
PIAS	protein inhibitor of activated STAT
PTEN	phosphatase and tensin homolog

qPCR	quantitative PCR
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SH2	Src-homology-2
SHP	Src-homology containing phosphatase
SOCS	suppressor of cytokine signaling
SPARCL-1	secreted protein acidic rich in cysteine like-1
STAT	signal transducer and activators of transcription
TA	transcriptional activation
USF	upstream transcription factor
VEGF	vascular endothelial growth factor
WB	western blot

Abstract

ANALYSIS OF NFI-X3 AND STAT3 INTERACTION AND ITS FUNCTION

By Etsegenet Tizazu, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Masters of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Tomasz Kordula

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YKL-40 is a secreted protein that is highly up-regulated in malignant glioblastoma (GBM). Its expression is correlated with the invasive nature of GBMs and poor diagnosis of patients (Nigro et al., 2005). Previous research has shown that in astrocytes and GBM cells, YKL-40 expression is regulated by two transcription factors, NFI-X3 and STAT3, which form a complex with each other (Singh et al., 2011). Here, we show that the N-terminal domain of NFI-X3 is sufficient and required for its interaction with STAT3. We also show that the DNA-binding domain of NFI-X3 is required to induce YKL-40 expression. Thus, the interaction of NFI-X3 with STAT3 may play a role in stabilizing the

otherwise weak binding of NFI-X3 to the YKL-40 promoter. Collectively, the observations made in this study shed light on the mechanisms by which NFI-X3, in concert with STAT3 regulate YKL-40 expression.

CHAPTER 1: Analysis of NFI-X3 and STAT3 interaction and its function

Introduction

Glioblastoma:

Glioblastoma multiforme (GBM), also known as grade IV malignant glioma, is the most common primary brain tumor. In the United States, GBM affects 13,000 people each year (Ohgaki et al., 2005). These tumors are highly aggressive, infiltrative, and destructive, making current treatments, such as surgical resection, radiation and chemotherapy ineffective (Schwartzbaum et al., 2006). The average time of survival after diagnosis is 14 months (Stupp et al., 2005). Although the molecular signaling pathways implicated in this disease are not well understood, recent research has shown that several signaling pathways may be involved. These pathways include phosphoinositide-3 kinase, AKT, Ras, and mitogen-activated protein kinase (MAPK), and receptor tyrosine kinases, including epidermal growth factor receptor (EGFR) and the vascular endothelial growth factor receptor (VEGFR). All of these pathways contribute to the growth and promotion of glioblastoma (Rao et al., 2004 and Guha et al., 2004). Recently, genes that are glioblastoma targeted have been elucidated and include, Tp53 (gene encoding p53), Rb, and PTEN. Moreover, a secreted glycoprotein, YKL-40 is overexpressed in patients with GBM and is associated with the strong vascularized phenotype of GBM.

YKL-40:

YKL-40, a member of the mammalian chitinase-like proteins is highly expressed in many types of cancers, including glioblastoma. The YKL-40 gene located on chromosome 1q31-q32 is 8 kb long and consists of 10 exons (Rehli et al., 2003). It contains a single polypeptide chain of 383 amino acids (Hakala et al., 1993). YKL-40 is named for its three NH₂-terminal amino acids Tyrosine (Y), Lysine (K) and Leucine (L) and its molecular weight of 40 kDa. As a member of the glycosyl chitinase family 18, YKL-40 can bind to chitin of different lengths (Aronson et al., 1997). Chitinases contain three amino acids that are essential for their glycolytic activity: Asp, Glu and Asp. In YKL-40, the Glu residue is replaced by Leu and as a result, YKL-40 does not have chitinase activity (Renkema et al., 1998). YKL-40 contains two possible sites of glycosylation, but only the NH₂-terminal is glycosylated with two units of N-acetylglucosamine via $\beta(1-4)$ linkage. It contains a binding site for heparin and two binding sites for hyaluronan acid (Fusetti et al., 2003).

Many cells secrete YKL-40, including macrophages, chondrocytes and synovial cells (Krause et al., 1996, Hakala et al., 1993). YKL-40 is a growth factor for fibroblasts, synovial cells and chondrocytes (De Ceuninck et al., 2001, Recklies et al., 2002, Ling et al., 2004). In fibroblasts, YKL-40 initiates mitogen-activated protein kinase and PI-3-kinase signaling cascades leading to the phosphorylation of both ERK 1/2 and protein kinase B (AKT) (Recklies et al., 2002 and Ling et al., 2004). As this signaling cascade is

associated with the control of mitogenesis, it is indicative of a role of YKL-40 as an anti-apoptotic protein.

YKL-40 is one of the most highly expressed genes in GBM and is secreted *in vitro* by human glioma cell lines (Nigro et al., 2005). Even though its role in cancer is not yet fully understood, it is believed that YKL-40 may play a role in the proliferation and differentiation of malignant cells, protection of cancer cells from undergoing apoptosis, stimulating angiogenesis and tissue remodeling. In immortalized astrocytes, YKL-40 increases resistance to radiation and increased invasion *in vitro* (Nigro et al., 2005). YKL-40 also plays a role in angiogenesis by stimulating the migration and re-organization of vascular endothelial cells (Malinda et al., 1999). Recently, it was shown that YKL-40 can act as an independent angiogenic factor and can also up-regulate VEGF, resulting in a synergistic effect on angiogenesis. In addition, YKL-40-induced angiogenesis is dependent on the interaction between membrane receptors syndecan-1 (Syn-1) and integrin $\alpha v \beta 3$ (Francescone et al., 2011).

Moreover, mice lacking YKL-40 gene have impaired antigen induced Th2 responses with YKL-40 acting as a potent inhibitor of death-receptor induced inflammatory cell apoptosis. The expression of IL-4 and IL-13 was decreased in YKL-40 null animals challenged with antigen compared to sham treated animals (Lee et al., 2009).

Even though YKL-40 has recently received much attention as a diagnostic marker for many diseases, little is known about its regulation. The YKL-40 promoter contains binding sites for many transcription factors, including: PU.1, Sp1, Sp3, USF, AML-1 and

C/EBP (Rehli et al., 2003). Also, NF- κ B, specifically its p65 and p50 subunits, negatively regulates YKL-40 in glioma cells (Bhat et al., 2008). Furthermore, YKL-40 expression in the brain is regulated by Nuclear Factor I-X (NFI-X) and Signal Transducers and Activators of Transcription 3 (STAT3) (Singh et al., 2011).

Signal Transducers and Activators of Transcription (STAT):

Signal Transducers and Activators of Transcription proteins regulate many aspects of growth, survival and differentiation in cells. In the human genome, STATs are clustered on three chromosomes and consist of seven mammalian members (STAT1, 2, 3, 4, 5a, 5b and 6). STAT proteins are between 700-850 amino acids long and contain many functional domains. They consist of an N-terminal protein interaction domain, coiled-coil domain, DNA binding domain, linker domain, SH2 domain and transactivation domain. At the C-terminus, all STATs contain a conserved Tyr residue that upon phosphorylation, interacts with the SH2 domain and allows their dimerization. Most STATs, excluding STAT2 and STAT6, contain a second conserved Ser residue at the transactivation domain that can be phosphorylated. The N-terminus of STATs is involved in dimerization, tetramerization and protein-protein interactions (Vinkemier et al., 1998). The coiled-coil domain is involved in the interaction with other proteins and may play a role in receptor binding (Zhang et al., 2000). The DNA binding domain assumes an immunoglobulin-fold structure and is involved in nuclear translocation by maintaining proper conformation for importin binding (Ma et al., 2006). The linker domain is involved in transcriptional

activation (Yang et al., 1999) and protein-protein interaction (Lufei et al., 2003). The SH2 domain, the most conserved domain, is critical for receptor association and phospho-dimer formation. The least conserved transactivation domain is involved in protein-protein interaction with CBP (p300)-binding protein and many other proteins, which leads to the specific activation of transcription (Zhang et al., 1996). STAT1 and STAT2 dimerize and can form a complex with IRF9 to bind the Interferon Sensitive Response Element (ISRE), while all other STATs bind to the Gamma interferon Activation Site (GAS) element.

STATs are activated by many cytokines, growth factors and hormones. STAT1 activation is induced by IFN- $\alpha/\beta/\gamma$. STAT1 is involved in anti-viral and anti-bacterial response, growth inhibition, apoptosis and tumor suppression. STAT2 is involved in IFN response. STAT3 is activated by cytokines and growth factors, such as IL-6 family members and EGF. STAT3 functions in wound-healing, regeneration of liver, mammary involution and survival of many cell types and is the only STAT that is required for early development (Takeda et al., 1997, Akira, 2000 and Poli et al., 2003). It is involved in survival, anti-apoptosis, mitogenesis and oncogenesis (Bromberg, 2001). STAT4 is induced by IL-12 and is involved in Th1 development, whereas STAT6 is involved in Th2 development and is activated by IL-4. STAT5a is involved in prolactin hormone signaling and STAT5b is involved in growth hormone signaling.

Upon activation, STATs are Tyrosine phosphorylated, which is important for their dimerization, nuclear import, DNA binding and transcriptional activation. This activation can take place by many mechanisms. Binding of cytokines leads to dimerization of the

cytokine receptors, which then leads to activation of the receptor-associated tyrosine kinase, Janus Kinases (JAKs). JAKs then trans-phosphorylate Tyr residues in the intracellular domain of the receptor. The phosphorylated Tyrosines provide a binding scaffold for STATs, which are recruited to the receptor via their SH2 domains. JAKs then phosphorylate STATs on a specific Tyr residue on the cytoplasmic tail, allowing them to form homo- or hetero- dimers by reciprocal binding of the phospho-Tyr to the SH2 domain of the partner. Upon dimerization, STATs dissociate from the receptor, translocate to the nucleus by associating with importins, bind DNA and activate the transcription of cytokine-responsive genes. Another mode of activation is with receptors that have intrinsic tyrosine kinase activities, which activate STATs directly. Yet another mechanism is via non-receptor tyrosine kinases, often used by viral oncoproteins that lead to constitutive activation of STATs.

Inactivation of STATs is regulated by many mechanisms, including the cytokine-inducible SH2-containing protein (CIS), suppressor of cytokine signaling (SOCS), STAT inhibitor (SSI), protein inhibitor of activated STAT (PIAS) and phosphatases as well as the ubiquitin-proteasome pathway (Kim, 1996). The family of SOCS/CIS proteins suppresses cytokine signaling by several mechanisms including competition for phospho-Tyr binding and promoting degradation of signaling molecules via ubiquitination (Starr et al., 2003), whereas the PIAS family interact directly with phosphorylated STATs in the nucleus and block their binding to DNA.

STATs play a central role in determining immune responses in cytokine-dependent inflammation and immunity. These proteins may control whether immune responses in the tumor environment promote or inhibit cancer. While STAT1, STAT5 and STAT6 are involved in inhibiting anti-tumor immunity, STAT3 regulates genes that are involved in tumor proliferation, survival, angiogenesis and invasion (Yu et al., 2007). STAT3 activation promotes tumor growth by antagonizing NF- κ B and STAT1-mediated anti-tumor response and mediating T regulatory cell expansion and TH17 development (Wang et al., 2009; Wu et al., 2009). STAT3 induces the expression of cytokines and chemokines, such as COX2 and IL-6 that are associated with cancer-promoting inflammation. Thus, STAT3 forms an autocrine and paracrine feed-forward loop that results in promotion of cancer. Additionally, STAT3 is constitutively activated in almost all human glioblastoma multiforme tissue. Furthermore, GBMs have higher expression levels of IL-6 and OSM, leading to constant activation of STAT3 signaling (Lim and Cao, 2006).

Nuclear Factor I (NFI):

The Nuclear Factor I family of transcription factors constitutes of four DNA-binding proteins, NFI-A, NFI-B, NFI-C, and NFI-X, which regulate the transcription of many cellular and viral genes as well as viral DNA replication. They are encoded by four highly conserved genes: *Nfia*, *Nfib*, *Nfic*, and *Nfix*. NFI proteins form homo- and heterodimers to bind the consensus sequence TTGGC(N)₅GCCAA on duplex DNA (Henninghausen et al., 1985, Leegwater et al., 1985, and Nowock et al., 1985).

In vertebrates, the four NFI genes are alternatively spliced yielding multiple mRNA and proteins from each gene. However, all isoforms of NFIs contain a conserved N-terminal DNA-binding and dimerization domain. The DNA-binding and dimerization domain is 200 amino acids long and is highly conserved within the four genes. This domain is enough for DNA-binding, dimerization and stimulation of adenoviral DNA replication (Mermod et al., 1989, Gounari et al., 1990) and is preceded by exons encoding 8-47 amino acid domain of unknown function, but is strongly conserved between the four genes (Meisterernst et al., 1989). Point mutations made in this region have shown that dimerization is needed for DNA-binding activity; however, DNA-binding ability can be eliminated independently of dimerization activity (Armentero et al., 1994). The N-terminal 1/3 of the DNA-binding and dimerization domain contains a highly basic alpha-helix. This helix consists of Lysine and arginine residues spaced at seven amino acid intervals (Meisterernst et al., 1989). The C-terminal 2/3 of the DNA-binding and dimerization domain contains four cysteine residues that are conserved between all NFI DNA-binding domains. Three of these cysteines (Cys 104, 120, and 163 in human NFI-C) are required for DNA-binding (Novak et al., 1992). The fourth cysteine (Cys 157) is important for protection to oxidative damage, but is not needed for DNA-binding activity (Bandyopadhyay et al., 1998).

The N-terminal domain is responsible for the DNA-binding and replication activities of NFI proteins. On the other hand, the C-terminal domain of NFI proteins regulates gene expression. Alternative splicing generates many variants of the C-terminal domains; however, cloning and characterization of NFI-C transcripts show that residues

399-499 are required for maximal transcriptional activity. This region is rich in proline residues and when linked to DNA-binding domains can induce a five-to-ten fold increase in transcription (Mermoid et al., 1989). Apart from its transcriptional activation, the C-terminal domain also includes a repression domain that inhibits transcription from many promoters, including GLUT4 genes (Adams, et al., 1995). Although the exact mechanism of repression is not known, direct competition with transactivators for binding has been shown (Nehls et al., 1991, 1992).

During embryogenesis of mammals, the NFI genes are expressed in overlapping patterns. NFI-A, -B and -X are highly expressed in the neocortex (Chaudhry et al., 1997). As a result, knockout of *Nfia* and *Nfib* genes in mice caused neuroanatomical defects, including a 5-10 fold decrease in the expression of astrocyte markers including Glial Fibrillary Acidic Protein (GFAP) (Shu et al., 2003, Steele-Perkins, 2005 and Neves, das L., 1999). Additionally, *Nfib* deficient mice die due to defects in lung development along with abnormal hippocampus and pons formation (Steele-Perkins et al., 2005). *Nfic* gene knockout causes early postnatal tooth formation defects (Steele-Perkins et al., 2003). *Nfix* gene knockout causes hydrocephalus and agenesis of the corpus collosum and leads to postnatal lethality in most animals (Driller et al., 2007). These knockout phenotypes of *Nfia*, *Nfib*, and *Nfix* indicate their roles in normal brain development. NFI-C and -X regulate expression of astrocyte markers during differentiation of neural precursors *in vitro* (Wilczynska, 2009). However, these transcription factors also play a role in abnormal brain physiology, such as gliogenesis (Deneen et al., 2006).

Recently, our lab has cloned and analyzed a novel splice variant of the *nfix* gene, NFI-X3. This variant contains a unique transcriptional activation (TA) domain completely conserved in primates. Compared to NFI-X1, the overexpression of NFI-X3 more potently activates NFI reporters, including GFAP reporter, in astrocytes and glioma cells. NFI-X3 expression is upregulated during the differentiation of neural progenitors to astrocytes and precedes the expression of astrocyte marker, GFAP and Secreted Protein Acidic Rich Cystein Like-1. The overexpression of NFI-X3 upregulates the expression of these marker genes in astrocytes and glioma cells. The TA domain of NFI-X3 activates GFAP expression by causing changes in the architecture of the +1 nucleosome that lead to the increased recruitment of RNA polymerase II (Singh et al., 2011). Additionally, our lab identified YKL-40 as a target gene of NFI-X3 in astrocytes and glioma cells that controls their migration. YKL-40 expression is activated during mouse brain development and also during the differentiation of neural progenitors into astrocytes *in vitro*. In primary astrocytes, YKL-40 expression is also controlled by STAT3. The knock-down of NFI-X and STAT3 significantly reduced YKL-40 expression in astrocytes, while overexpression of NFI-X3 induced YKL-40 expression in glioma cells. In addition, we have found that STAT3 and NFI-X3 activate YKL-40 expression via their binding site located in the YKL-40 proximal promoter (Singh et al., 2011). Moreover, we have found that NFI-X and STAT3 physically interact and it is possible that this complex may regulate YKL-40 expression.

Hypothesis

STAT 3 and NFI-X3 control GFAP expression both in astrocytes and GBM cells. Both of these transcription factors bind to their corresponding regulatory elements on the enhancer region and cause the eviction of nucleosome of the GFAP promoter and induce its expression (Singh et al., 2011). However, how these transcription factors regulate YKL-40 expression is not known. Our lab showed that a YKL-40 reporter gene lacking four NFI elements is fully activated by Oncostatin M, whereas the deletion of the proximal STAT element (-78 to -69) significantly reduces reporter activity. Furthermore, NFI-X3 and STAT3 weakly bind to their juxtaposed elements in the YKL-40 promoter *in vitro* and STAT3 co-immunoprecipitates with NFI-X3 from U373 cells (Singh et al., 2011). Thus, we hypothesize that STAT3 may recruit NFI-X3 to the YKL-40 promoter. In fact, we suspect that there is a direct interaction between STAT3 with NFI-X3 that is needed for the regulation of YKL-40 expression. Therefore, we hypothesize that this complex regulates a subset of genes, as is true for the NF- κ B/STAT3 complex (Yang J. et al., 2007 and Yu et al., 2002). The aim of this thesis is to identify the specific regions in NFI-X3 that are required for its interaction with STAT3 and show that the NFI-X3/ STAT3 complex regulates a subset of genes, including YKL-40.

Materials and Methods

Cell culture and reagents: Human glioblastoma U373-MG cells and human embryonic kidney HEK293 cells were provided by American Type Culture Collection (Rockville, MD). These cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, antibiotics, and non-essential amino acids.

Plasmids and site-directed mutagenesis: pSTAT3-V5 and vector expressing full-length NFI-X3FLAG was obtained from Singh et al., (2011). The following deletions and point mutations of NFI-X3FLAG were made using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) following their protocol. The above vector was used as a template and primers were ordered from Eurofins MGE Operon, Huntsville, AL. The forward 5'-CACCGGACAGAGGTCCGGAACCGATTACAAGGACGACG- 3' and the reverse 5'-GGTTCCGGACCTCTGTCCGGTGGCCTGG-3' primers were used to delete 84 amino acids from the C-terminus of NFI-X3FLAG (NFI-X3FLAG-del 1). Plasmid containing deletion was analyzed with AccIII. The forward, 5'- TGTGGATGCAAGGTC CGGAACCGATTACAAGGACGACG-3' and the reverse, 5'-GGTTCCGGACCTTGCA TCCACATCGTTGG-3' primers were used to delete 184 amino acids from the C-terminus (NFI-X3FLAG-del 2). Plasmid containing this deletion was analyzed with BstXI. The forward, 5'- TTTTGTGACTAGGTCCGGAACCGATTACAAGGACGACG-3' and the reverse, 5'- GGTTCCGGACCCTAGTCACAAAACAGTCCTGG-3' primers were used to delete 284 amino acids from the C-terminus (NFI-X3FLAG-del 3). Plasmid containing this deletion was analyzed with AccIII. The NFI-X3FLAG N-terminus α -helix deletion

(NFI-X3FLAG-NT del) was made using the forward, 5'-TCGCCATGGATGAGTTCCGCGAGGACTTCG-3' and the reverse, 5'-TCGCGGAACTCATCCATGGCGAAGCTTGAATTC-3' primers. The plasmid containing this deletion was analyzed with Hind III. The NFI- X3FLAG C148L, C154L (NFI-X3FLAG-C148L, C154L) point mutations were made by using the forward, 5'-CTCAGCTGTCGAACCCCGGCCTGCTCGTCCAGCCACATCACATTG-3' and the reverse, 5'-TGGACGAGCAGGCCGGGGTTCGACAGCTGAGGCGACTTGTAGAGC - 3' primers. The plasmid containing these point mutations was analyzed with PvuII.

Transient Co-transfections: HEK293 cells were transiently transfected in 6 well clusters using Lipofectamine LTX and PLUS Reagent (Invitrogen, Carlsbad, CA) using the supplier's protocol. On day 1, cells were transfected with 1 µg of NFI-X3FL, NFI-X3FL-del 1, NFI-X3FL- del 2, NFI-X3FL- del 3, NFI-X3FL-NT del and NFI-X3FL- C148L, C154L mutation. On day 2, cells were transfected with 1 µg of pSTAT3-V5. Two days after the initial transfection, cells were harvested, protein extracts were prepared, and protein concentration was determined by the BCA method (Sigma Chemical Co., St. Louis, MO).

Stable Transfections: Stable clones were generated in U373 cells by using 4 µg of NFI-X3FL- C148L, C154L plasmid and 0.2 µg of a second plasmid containing either zeocine or hygromycin resistance gene. Cells were transfected in 10 cm dishes and clones were selected in DMEM containing 100 µg/ml hygromycin and 300 µg/ml zeocine.

Western blotting: Cells were lysed in 1 ml of IP buffer (10 mM Tris 7.4, 150 mM sodium chloride, 1 mM EDTA, 0.5% NP-40, 1% Triton X-100, 1 mM sodium orthovanadate, and protease inhibitor cocktail (Roche, Mannheim, Germany)). Samples were resolved using SDS-PAGE and electroblotted onto nitrocellulose membrane (Schleicher & Schuell, Keene, NH). The blots were incubated with an anti-FLAG antibody (0.3 µg/ml) or an anti-V5 antibody (1.2 mg/ml) overnight at 4°C. The blots were washed extensively with a solution containing 0.15M NaCl, 0.01M Tris pH 8.0, and 1% NP-40. The anti-FLAG antibody was purchased from Sigma-Aldrich, St. Louis, MO. The anti-V5 antibody was purchased from Invitrogen, Carlsbad, CA. Anti-mouse secondary antibody was used in both cases and antigen-antibody complexes were visualized by enhanced chemiluminescence using Immobilon Western blotting kit (Millipore, Temecula, CA).

Co-Immunoprecipitation: Protein lysates were prepared as described in western blotting section. 20 µg/ml of FLAG beads (Sigma –Aldrich, St. Louis, MO) was added to tubes containing 200-300 µg of protein lysates and 1 ml of IP buffer and incubated overnight at 4°C with rocking. Immunoprecipitates were washed three times with 1 ml of IP buffer and once with 900 µl of 1X TBS (50 mM Tris pH 7.4, 150 mM NaCl). Complexes were eluted using 15 µg of FLAG peptide and analyzed by SDS/PAGE (10% gels) followed by western blot.

RNA isolation and quantitative PCR: Total cellular RNA was prepared using Trizol (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Subsequently, 1 µg of total RNA was reverse-transcribed using the High Capacity cDNA Archive kit (Applied

Biosystems, Foster City, CA). YKL-40, GFAP, and GAPDH mRNA levels were measured using pre-mixed primer-probe sets, and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The cDNAs were diluted 10-fold for the GFAP and YKL-40 genes and 100-fold for GAPDH and amplified using the ABI 7900HT cyclor. Gene expression levels were normalized to GAPDH mRNA levels, and presented as a fold induction with mean +/- standard deviation.

Nuclear Localization: HEK293 cells were transfected with 1 μ g of NFI-X3FL, NFI-X3FL- del 1, NFI-X3FL- del 2, NFI-X3FL- del 3, NFI-X3FL-NT del and NFI-X3FL-C148L, C154L mutations using Lipofectamine LTX and PLUS Reagent (Invitrogen, Carlsbad, CA) using the supplier's protocol. Twenty-four hours after transfection, cells were washed with non-sterile PBS three times, and fixed with 3% paraformaldehyde in 1ml for 30 minutes at room temperature. After PBS washing twice, cells were permeabilized with 0.1% Triton-X for 20 minutes at room temperature. After PBS washing twice, anti-FLAG (M2) (Sigma-Aldrich, Saint Louis, MO) antibody was prepared in 5% ELISA grade BSA (1:100 dilution) and added to cells, which were then incubated at 4°C overnight under moist conditions. Next day, cells were washed with PBS twice and Alexa Fluor® 488 anti-mouse IgG (Invitrogen, Carlsbad, CA) was used as a secondary antibody (1:400 dilution), and cells incubated for thirty minutes in the dark. After washing with PBS twice, Hoescht (50ng/ml) was added to the cells and incubated at room temperature for 3 minutes. Cells were then washed with PBS twice. After cells were mounted, they were visualized with Fluorescence microscopy and photographs were taken using Nikon Eclipse E1000 camera.

Results

Mutations of NFI-X3 domains.

In order to understand which domain of NFI-X3 is important for its interaction with STAT3, deletion mutants were constructed using synthetic oligonucleotides (Figure 1 and 6). Three constructs were generated in which amino acids were deleted from the C-terminus: NFI-X3FL-del 1 lacked 84 amino acids (411-495), NFI-X3FL-del 2 lacked 184 amino acids (311-495) and NFI-X3FL-del 3 lacked 284 amino acids (211-495). To assess the proper generation of these deletion mutants, digestions with restriction enzymes were carried out and fragments visualized on 2% agarose gel with EtBr. In some cases, full-length NFI-X3FL was digested as a control. The sizes of the fragments were predicted by maps created using the ApE program. NFI-X3FL-del 1 was digested with AccIII. The full-length plasmid showed a fragment of 6108 bases, whereas del 1 showed two fragments, 696 and 5160 bases (Figure 2). NFI-X3FL-del 2 plasmid was digested with BstXI. The full length plasmid showed two fragments, 5072 and 1040 bases, whereas del 2 showed fragments of 5074 and 489 bases (Figure 3). NFI-X3FL-del 3 was digested with AccIII and showed two fragments of 5160 and 96 bases (Figure 4).

Figure 1: C-terminus deletions of NFI-X3FL. Synthetic oligonucleotides were used to create three deletion mutants of NFI-X3FL. These mutants were created using the NFI-X3FL as a template and using the QuikChange® Site-Directed Mutagenesis kit and protocol. These mutants lack 84 amino acids (NFI-X3FL-del 1), 184 amino acids (NFI-X3FL-del 2) and 284 amino acids (NFI-X3FL-del 3) from the C-terminus, as depicted below. NFI-X3 consists of N-terminal α -helix, DNA-binding domain as well as four conserved Cysteines within DNA-binding domain. On the C-terminus, NFI-X3 contains a transcriptional activation (TA) domain.

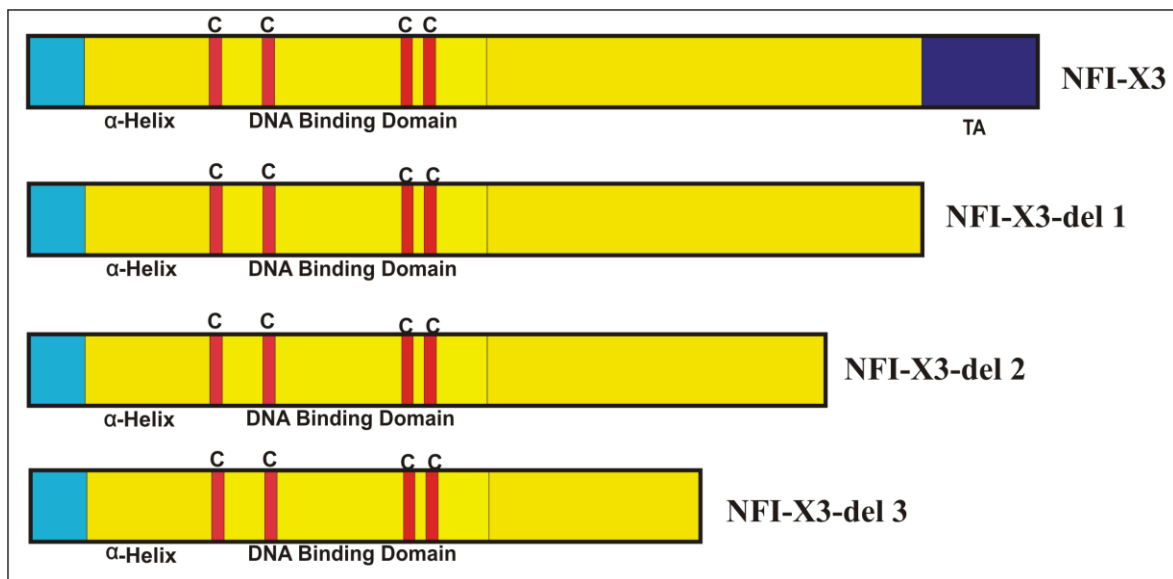


Figure 2: NFI-X3FL-del 1 plasmid digestion with AccIII. Synthetic oligonucleotides were used to delete 84 amino acids from the C-terminus of NFI-X3FL using the full-length NFI-X3FL as a template and following QuikChange® II Site-Directed Mutagenesis Kit protocol. Plasmid containing this deletion was purified and digested with AccII. Digestion map showing the sizes of the predicted fragments of full-length and NFI-X3FL-del 1 were created using the ApE program.

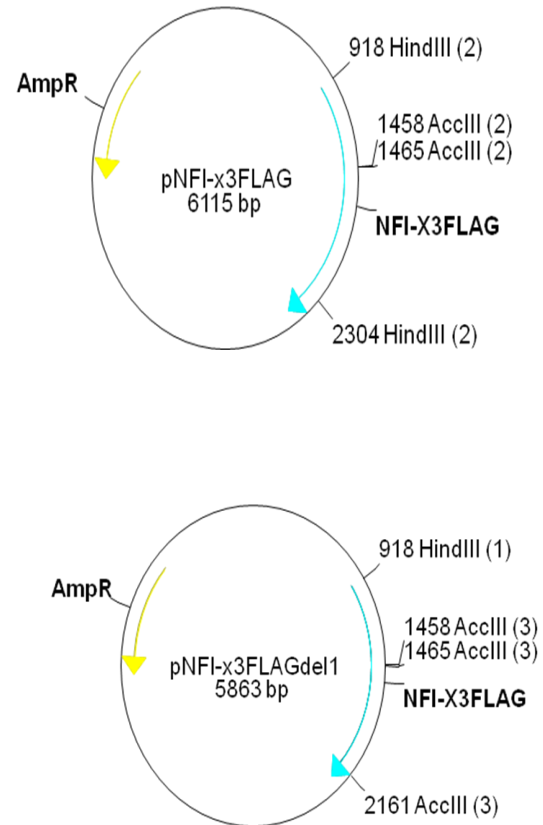
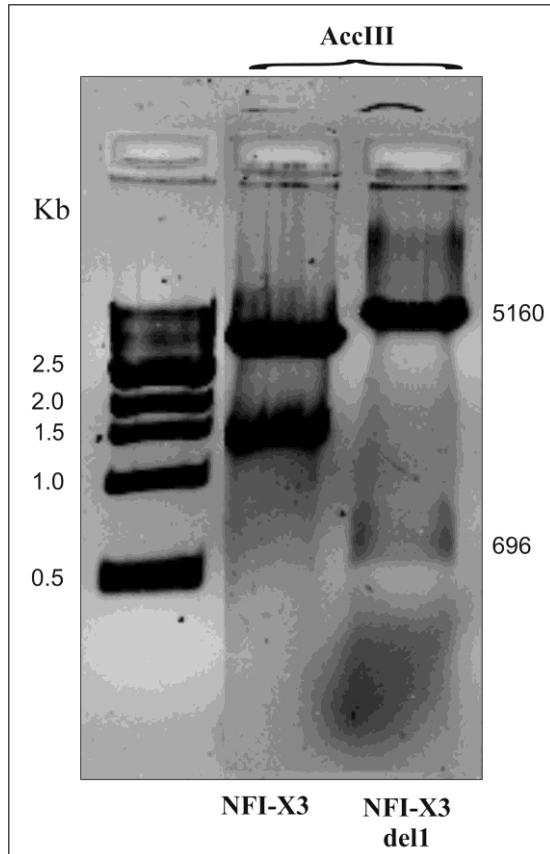


Figure 3: NFI-X3FL-del 2 plasmid digestion with BstXI. Synthetic oligonucleotides were used to delete 184 amino acids from the C-terminus of NFI-X3FL using the full-length NFI-X3FL as a template and following QuikChange® II Site-Directed Mutagenesis Kit protocol. Plasmid containing this deletion was purified and digested with BstXI. Digestion map showing the sizes of the predicted fragments of full-length and NFI-X3FL-del 2 were created using the ApE program.

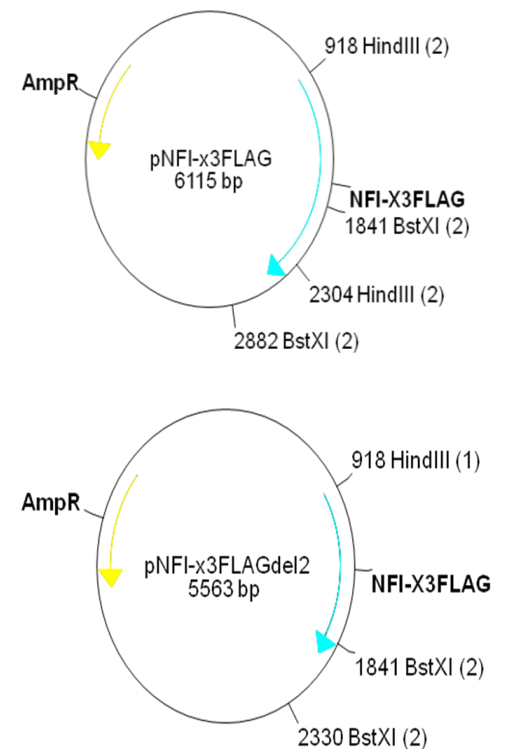
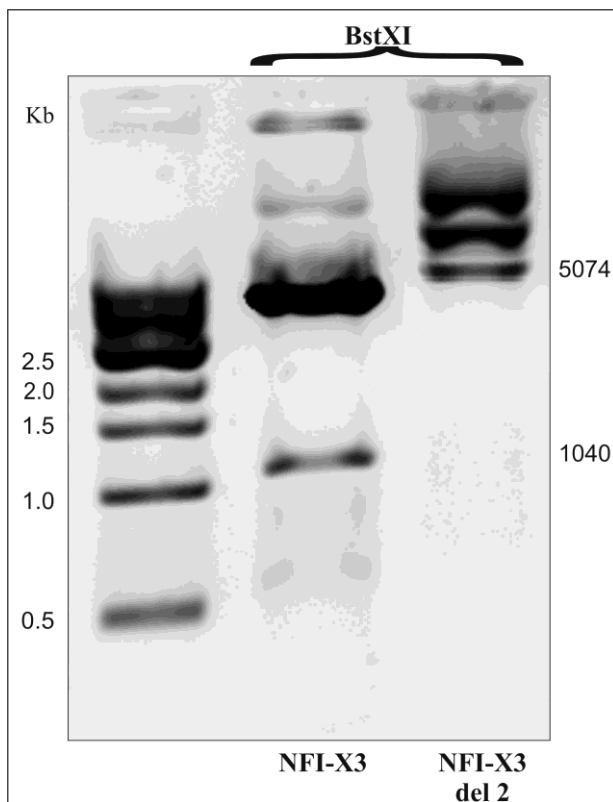
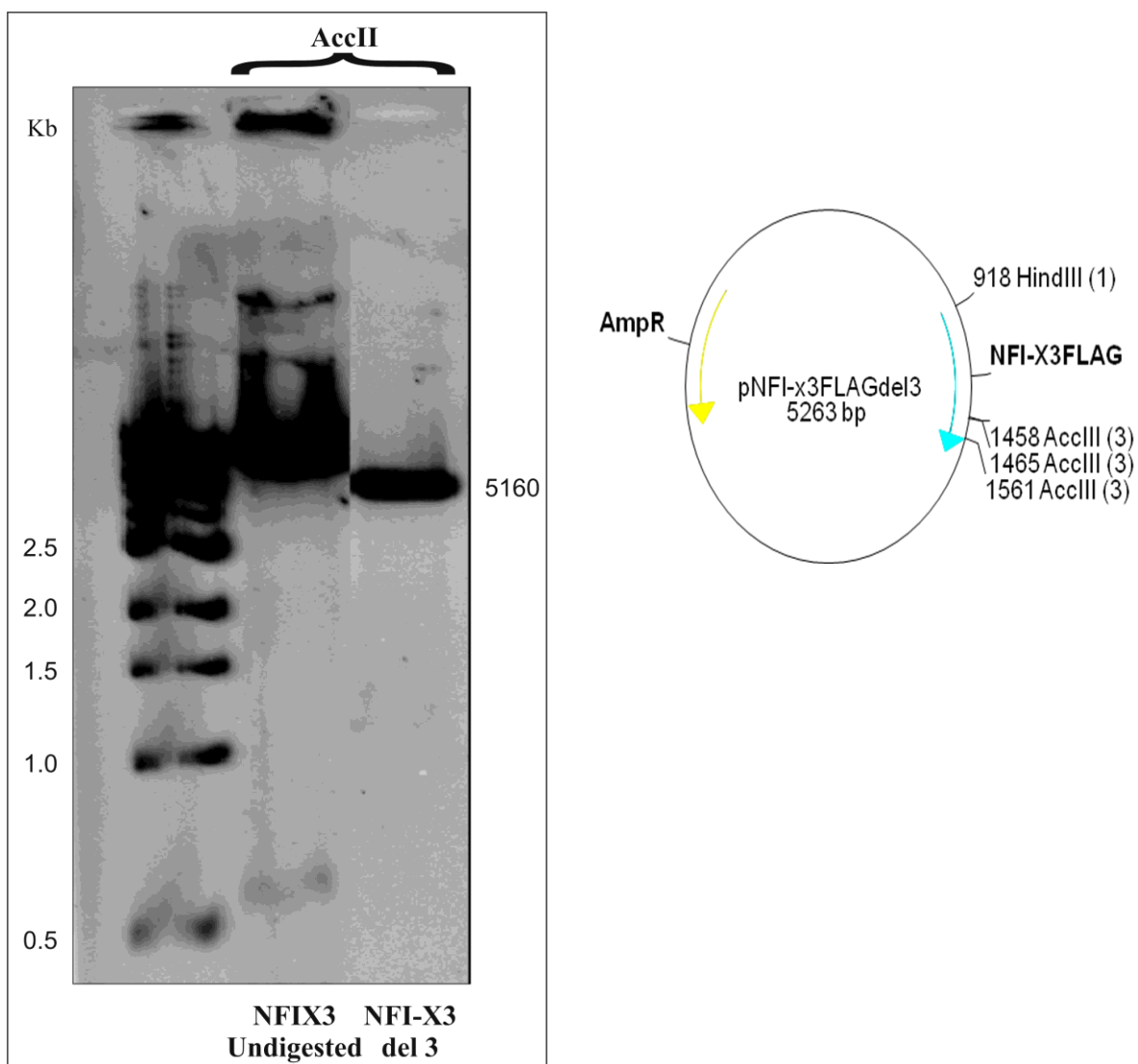


Figure 4: NFI-X3FL-del 3 plasmid digestion with AccIII. Synthetic oligonucleotides were used to remove 284 amino acids from the C-terminus of NFI-X3FL using the full-length NFI-X3FL as a template and following QuikChange® II Site-Directed Mutagenesis Kit protocol. Plasmid containing this deletion was purified and digested with AccIII. Digestion map showing the sizes of the predicted fragments of NFI-X3FL-del 3 was created using the ApE program.

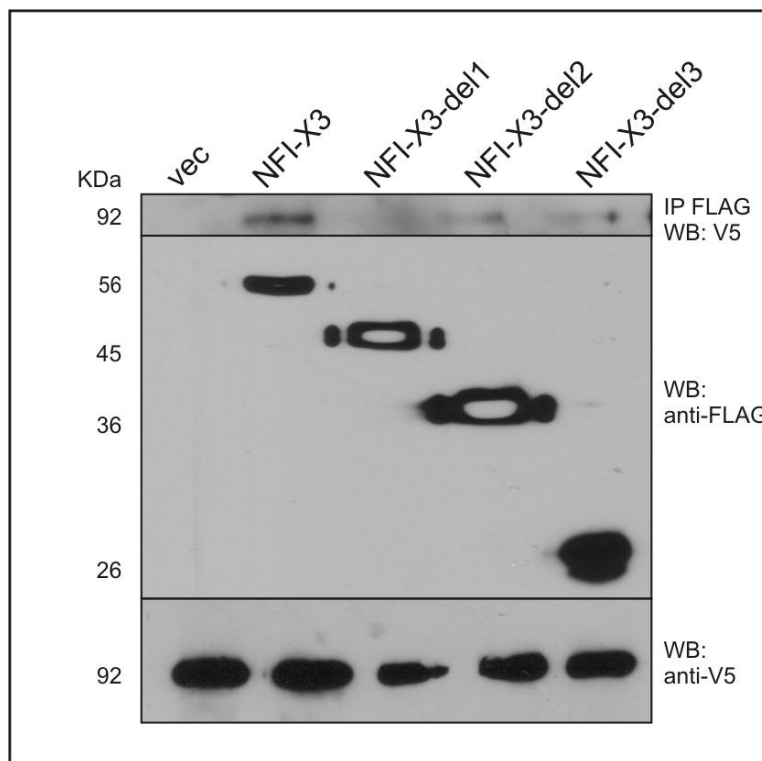


The N-terminus of NFI-X3 is sufficient for its interaction with STAT3.

In order to investigate if the C-terminus of NFI-X3 was needed for its interaction with STAT3, an empty vector, full-length NFI-X3FL, NFI-X3FL-del1, NFI-X3FL-del 2, and NFI-X3FL-del 3 were transiently transfected in HEK cells along with STAT3-V5. Cells were lysed and run on SDS-PAGE and expression of both STAT-V5 (92 kDa) (Figure 5C) and NFI-X3FLAG (Figure 5B) was analyzed by Western Blot. Figure 5B shows the expression of the NFI-X3FL constructs: full length NFI-X3FL (56 kDa), del 1 (45 kDa) , del 2 (36 kDa) and del 3 (26 kDa). The same lysates were used to perform Co-Immunoprecipitation. Samples were then run on SDS-PAGE and result analyzed by Western Blot (Figure 5A). Results show that with similar expression of both STAT3-V5 and NFI-X3FL, all three deletions are still able to interact with STAT3. However, the interaction is weaker in cells containing the deletion mutants when compared to those transfected with full- length NFI-X3.

Figure 5: The N-terminus of NFI-X3 is sufficient for its interaction with STAT3.

HEK293 cells were co-transfected with 1 μ g of STAT3-V5 and 1 μ g of empty vector, full-length NFI-X3 or NFI-X3 containing either del 1, del 2 or del 3. 20 μ g of proteins from SDS-PAGE Then lysates using FLAG beads and STAT3-V5 was detected by western blotting using anti-V5 antibodies (A). The expression of STAT3-V5 (C), NFI-X3FL and the NFI-X3FL C-terminus deletion mutants (B) were determined in total lysates by western blotting.



The N-terminus alpha-helix is required for NFI-X3 and STAT3 interaction.

Co-Immunoprecipitation done with STAT3-V5 and C-terminus deletions of NFI-X3FL showed that the N-terminus is sufficient for the interaction between STAT3 and NFI-X3 (Figure 5A). Thus, the N-terminal alpha-helix was deleted (Figure 6). This deletion mutant, NFI-X3FL-NT del, was made by deleting the N-terminal alpha helix (Figure 6). Plasmid containing this deletion was digested with HindIII and showed fragments of 4729 and 1161 bases (Figure 7). HEK293 cells were then co-transfected with 1 µg of NFI-X3FL-NT del and 1 µg of STAT3-V5 plasmids. . Cells transfected with pFLAG5A was used as a negative control and cells transfected with NFI-X3FL served as a positive control. After cells were lysed, they were run on SDS-PAGE and expression of both plasmids was checked via Western Blot analysis. Indeed, these cells do express both STAT3-V5 (92 kDa) (Figure 8C) and NFI-X3FL-NT del (46 kDa) (Figure 8B). Then, Co-IP was performed and samples run on SDS-PAGE and analyzed by Western Blot (Figure 8A). The results show that NFI-X3FL-NT del does not interact with STAT3. Hence, the alpha-helix is required for the interaction of NFI-X3 with STAT3.

Figure 6: NFI-X3FL-NT deletion. Synthetic oligonucleotides were used to create a deletion mutant of NFI-X3FL. This mutant was created using the NFI-X3FL as a template and using the QuikChange® Site-Directed Mutagenesis kit and protocol. In this mutant, the α -helical domain is deleted, as depicted below. NFI-X3 consists of N-terminal α -helix, DNA-binding domain as well as four conserved Cysteines within DNA-binding domain. On the C-terminus, NFI-X3 contains a transcriptional activation (TA) domain.



Figure 7: NFI-X3FL-NT del plasmid digestion with HindIII. Synthetic oligonucleotides were used to delete the alpha-helical domain of NFI-X3FL using the full length NFI-X3FL as a template and following QuikChange® II Site-Directed Mutagenesis Kit protocol. Plasmid containing this deletion was purified and digested with HindIII. Digestion map showing the sizes of the predicted fragments of NT del containing NFI-X3FL was created using the ApE program.

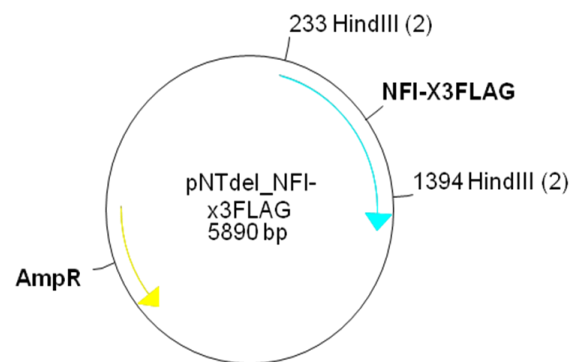
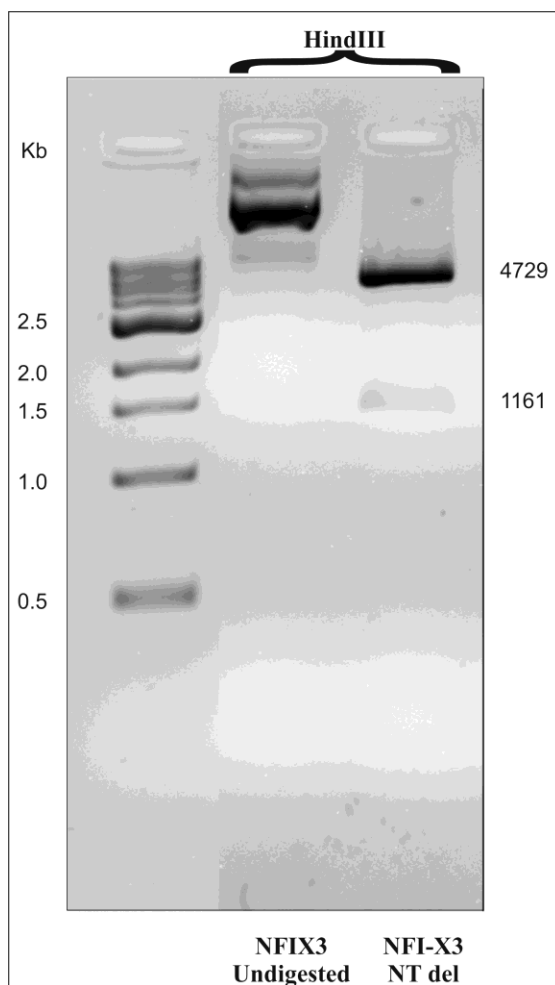
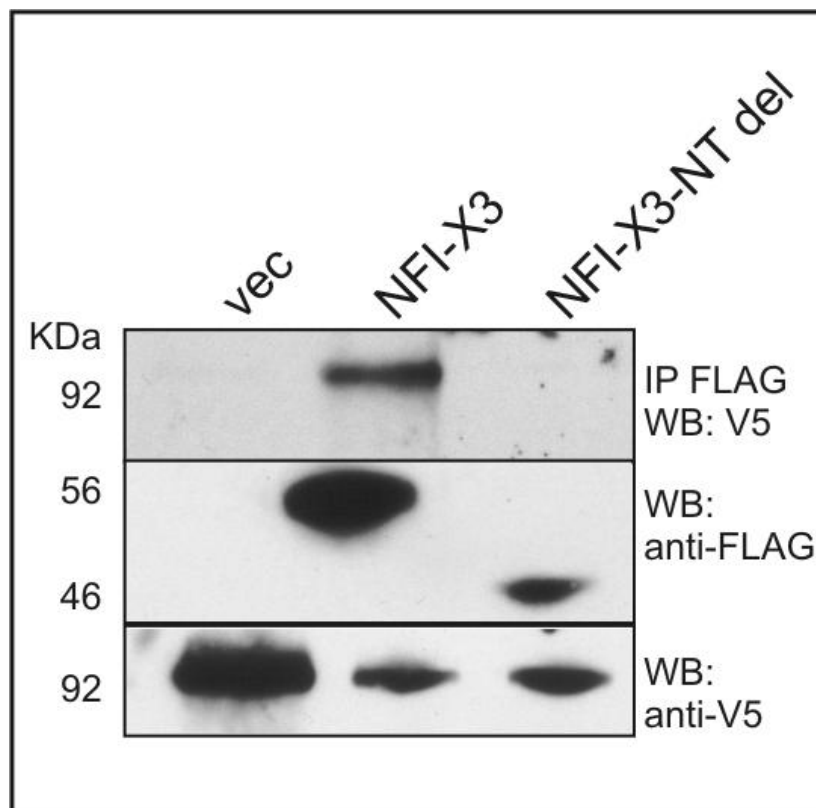


Figure 8: The α -helical domain of NFI-X3 is required for its interaction with STAT3.

NFI-containing complexes were immunoprecipitated from HEK293 cells co-transfected with 1 μ g of both STAT3 and empty vector, full-length NFI-X3 or NFI-X3 containing NT del using FLAG beads and STAT3-V5 was detected by western blotting using anti-V5 antibodies (A). The expression of STAT3-V5 (C), NFI-X3FL and the NFI-X3FL NT deletion mutants (B) were determined in total lysates by western blotting.



NFI-X3 forms a complex with STAT3 and regulates YKL-40 expression via direct binding.

Moreover, a construct containing point mutations of two conserved cysteine residues was made to assess whether NFI-X3 directly binds to the YKL-40 promoter to induce its expression in GBMs (Figure 9). This plasmid, NFI-X3FL-C148L, C154L mutation was digested with PvuII into four fragments, 2515, 1858, 1297 and 445 bases (Figure 10). U373 stable cells were created by co-transfecting the cells with 4 µg of NFI-X3FL- C148L, C154L mutation plasmid and 0.2 µg of a second plasmid containing either zeocine or hygromycin resistance gene. Cells were then selected in DMEM containing 100 µg/ml hygromycin and 300 µg/ml zeocine. Cells were then lysed and run on SDS-PAGE and analyzed by Western Blot for their expression of NFI-X3FL –C148L, C154L mutation (56 kDa) (Results not shown). Moreover, RNA was isolated from these cells using TRizol protocol (see materials and methods) and qPCR was performed to assess the expression of YKL-40 and GFAP (Figure 11). The expression of both GFAP and YKL-40 was significantly reduced in cells containing mutations in the DNA binding domain of NFI-X3 compared to cells that contained full-length NFI-X3 (Figure 11).

Figure 9: NFI-X3FL-C148L, C154L point mutations. Synthetic oligonucleotides were used to create a mutant of NFI-X3FL to abolish its DNA-binding ability. This mutant was created using the NFI-X3FL as a template and using the QuikChange® Site-Directed Mutagenesis kit and protocol. On the C-terminus, NFI-X3 contains a transcriptional activation (TA) domain. On the N-terminus, NFI-X3 contains an α -helix, DNA-binding domain as well as four conserved Cysteines within DNA-binding domain. Two of these conserved cysteines (C148 and C154) within the DNA binding domain were mutated to leucines, as depicted below.

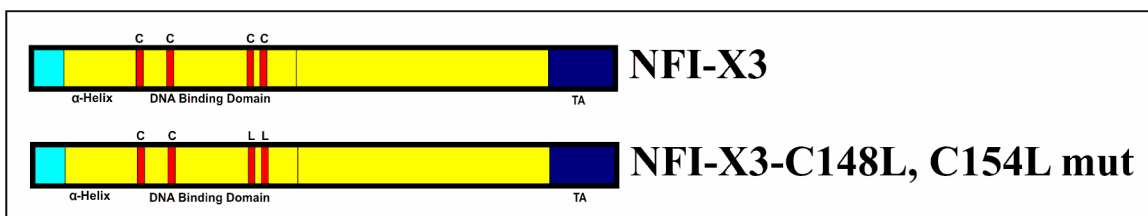


Figure 10: NFI-X3FL-C148L, C154L plasmid digestion with PvuII. Synthetic oligonucleotides were used to mutate C148 and C154 of NFI-X3FL to leucines using the full length NFI-X3FL as a template and following QuikChange® II Site-Directed Mutagenesis Kit protocol. Plasmid containing these point mutations was purified and digested with PvuII. Digestion map showing the sizes of the predicted fragments after digestion with PvuII was created using the ApE program.

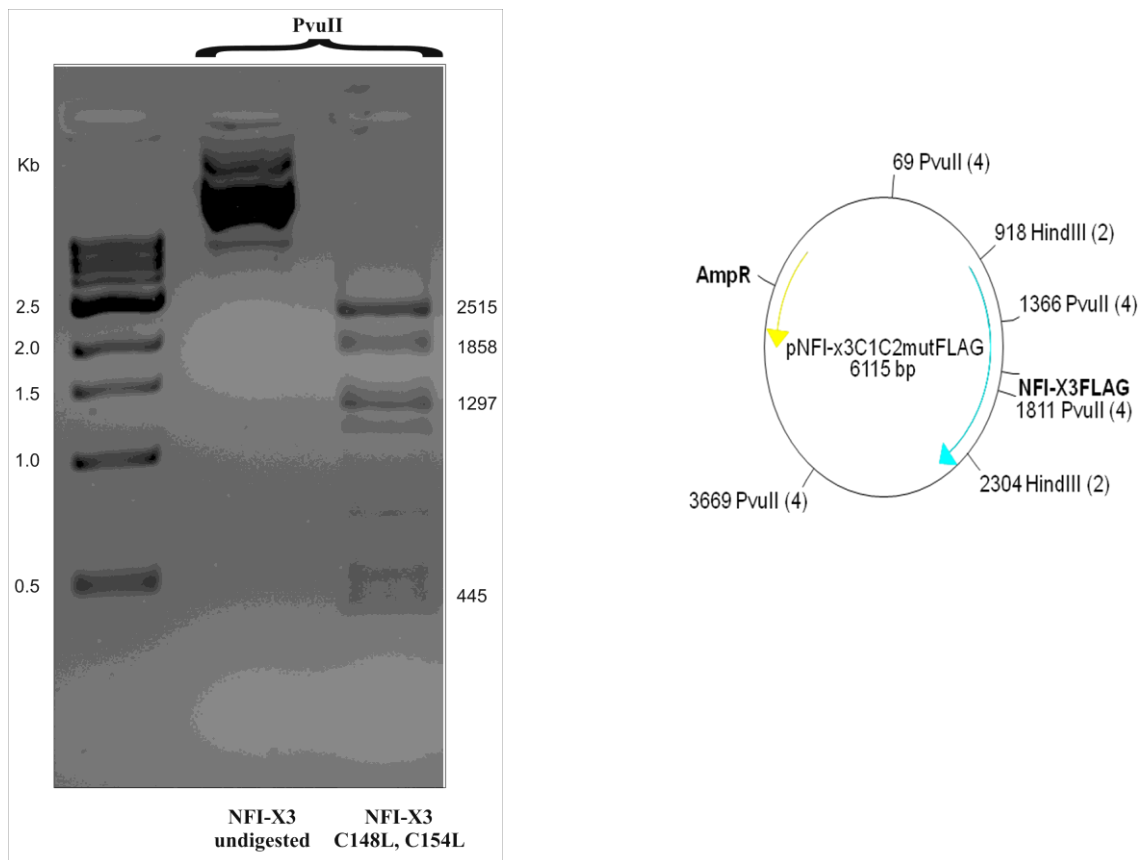
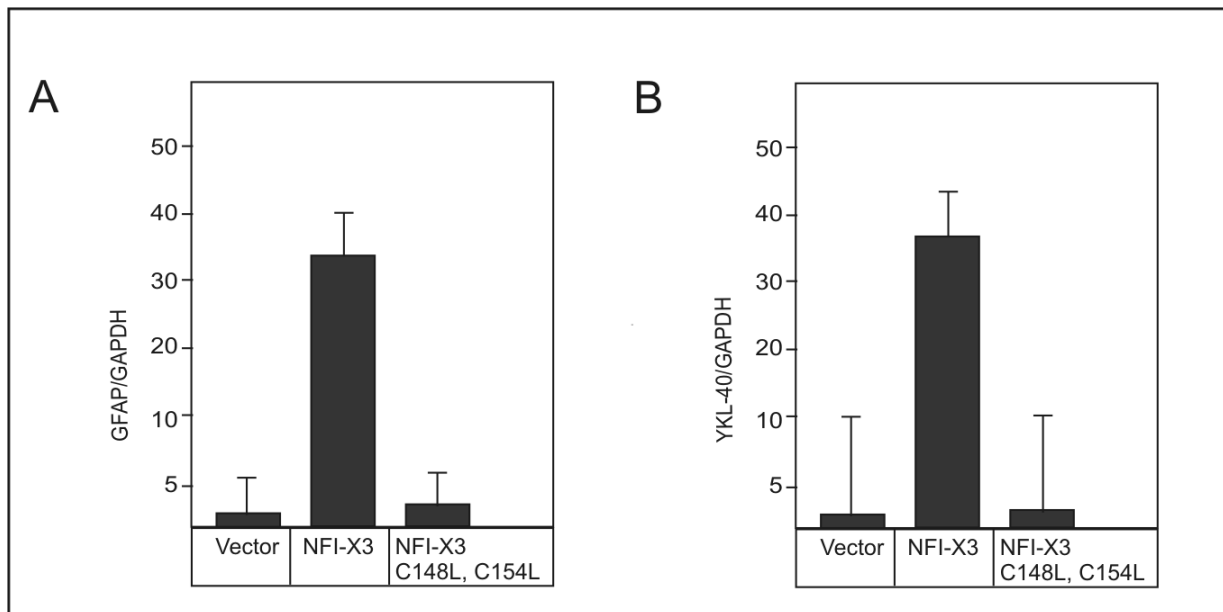


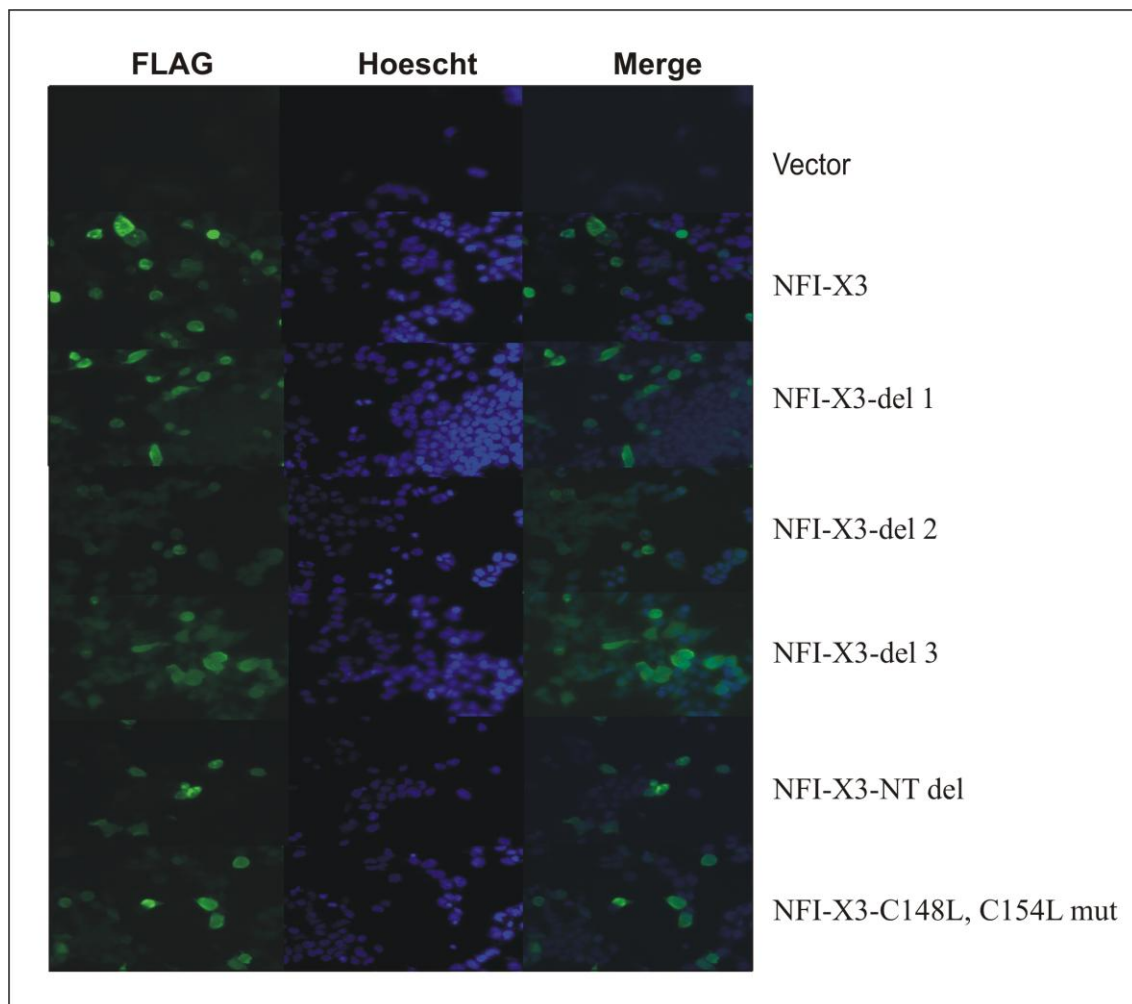
Figure 11: The DNA-binding domain of NFI-X3 is required to induce YKL-40 expression. Pools of U373 stable clones transfected with plasmids expressing either full-length NFI-X3 or NFI-X3-C148L, C154L mutant or empty vector were cultured as described in materials and methods. RNA was isolated from these cells and reverse transcribed, and the expression of GFAP (A) and YKL-40 (B) was analyzed by qPCR.



All NFI-X3FL constructs can translocate to the nucleus.

Staining was done to assess the ability of our NFI-X3FL deletion constructs to translocate to the nucleus. HEK cells were transfected with 1 µg of pFLAG5A vector, NFI-X3FL, NFI-X3FL-del 1, NFI-X3FL-del 2, NFI-X3FL-del 3, NFI-X3FL-NT del and NFI-X3FL-C148L, C154L mutation. Twenty-four hours after transfection, cells were washed and fixed onto cover slips and incubated overnight with anti-FLAG antibody (1:100 dilutions). The next day, cells were washed and incubated with Alexa Fluor® 488 anti-mouse IgG antibody (1:400 dilutions) for thirty minutes in the dark. The cells were then washed and incubated with Hoescht (50ng/ml) for 3 minutes, washed, and mounted onto slides and pictures taken. Cells that contain FLAG epitope are green and the nuclei are stained blue. Cells transfected with pFLAG5A was used as a negative control and cells transfected with NFI-X3FL served as a positive control. Figure 12 shows that NFI-X3FL-del 1 was mainly in the nucleus, while NFI-X3FL-del 2 and NFI-X3FL-del 3 were both cytoplasmic and nuclear. However, NFI-X3FL-del 2 was located mainly in the cytoplasm. NFI-X3FL-NT del was located both in the cytoplasm and nucleus, whereas NFI-X3FL-C148L, C154L mutation was mostly located in the nucleus.

Figure 12: All of the NFI-X3FL constructs can localize to the nucleus. Plasmids containing either full length NFI-X3FL or NFI-X3FL deletion mutants or empty vector were transfected into HEK293 cells and stained with Alexa Fluoro 488 anti-mouse IgG to recognize the FLAG epitope and Hoescht (similar to DAPI) for nuclear staining and visualized by Fluorescent Microscopy.



Discussion

Malignant glioblastoma (GBM) are highly invasive and resistant to radiation and other treatments. As a result, patients with GBMs have poor prognosis. Recent research has shown that a secreted ECM protein, YKL-40, is highly expressed in GBMs and that its high expression is associated with the GBMs' resistance to radiation as well as its invasive nature (Nigro et al., 2005 and Francescone et al., 2011). However, how this expression of YKL-40 in GBMs is regulated is not known. Recently, Singh et al. (2011) showed that YKL-40 is regulated by Nuclear Factor I-X3 (NFI-X3) and Signal Transducers and Activator of Transcription 3 (STAT3), which can form a complex. Moreover, Singh et al., (2011) showed that the binding of both STAT3 and NFI-X3 is required for the expression of the astrocyte marker, Green Fibrillary Acidic Protein (GFAP).

In this study, we set out to find the domains of NFI-X3 that are required for the formation of this complex. First, deletions were made by removing 84, 184 and 284 amino acids from the C-terminus of NFI-X3. Co-Immunoprecipitation experiments carried out with these deletions show that STAT3 and NFI-X3 do indeed form a complex and that, the N-terminus of NFI-X3 is sufficient for its interaction with STAT3 (Figure 5). However, the interaction is diminished in these deletions, suggesting that the C-terminus may stabilize this interaction. The C-terminal transcriptional activation (TA) domain is rich in prolines, which play an important role in regulating protein-protein interactions. Thus, these prolines might play a role in stabilizing the interaction between STAT3 and NFI-X3. To further elucidate which N-terminus domains are required, the alpha-helical domain of NFI-

X3 was deleted. Co-Immunoprecipitation with this deletion and STAT3 shows that the alpha-helical domain is indispensable for this interaction (Figure 8). NFI genes can undergo alternative splicing, affecting mostly their C-terminus, to generate transcription factors with diverse functions. Grunder et al., 2003 showed that splice variants of NFI-A, NFI-B and NFI-C are active transcription factors. It would be of great interest to assess whether these NFI-X3 C-terminus deletion mutants are active and if they regulate genes that are distinct from those controlled by NFI-X3.

We also show that all of the NFI-X3 constructs that we created are capable of translocating to the nucleus (Figure 12). NFI proteins contain two nuclear localization signals (NLS) located on exon 2 and exons 3-6. The NLS located on exon 2 (NLS1) is composed of 24 amino acids (aa 36-50), RKRKYFKKHEKRMSK while the NLS located on exon 3-6 (NLS2) is composed of 34 amino acids (aa 251-284), RRSITSPPSTSTTKRPK (Imagawag et al. 2008). Complete nuclear localization requires both signals, although, one signal is sufficient for some localization. NFI-X3FL-del 1, NFI-X3FL-del 2, and NFI-X3FL C148L, C154L mutation constructs contain both nuclear localization signals. However, NFI-X3FL-del 3 contains only NLS1, whereas NFI-X3FL-NT del contains only NLS2. As a result, these constructs do not localize to the nucleus as well as those that contains both NLS signals. Most importantly, the ability of these constructs to localize to the nucleus does not affect their capacity to interact with STAT3 as it shuttles between the cytoplasm and nucleus (Meyer and Vinkemeier, 2004) resulting in a steady state subcellular distribution that results from continuous nuclear import and export (Pranada et al., 2004).

We also wanted to assess whether NFI-X3 and STAT3 regulate YKL-40 expression by binding directly to the gene, like that shown for GFAP expression. In order to test this, NFI-X3FL containing point mutations within the DNA binding domain was created (Figure 9). Novak et al., (1992) showed that three (C104, 120 and 163) of the four conserved cysteine residues within the DNA binding domain of NFI-C are required for DNA binding. In our construct, Cys 148 and 154, corresponding to Cys 104 and 120, were mutated to leucines. Stably transfected U373 cells containing this construct show that the DNA binding domain of NFI-X3 is required to induce YKL-40 expression (Figure 11). This suggests that NFI-X3 binds directly to the YKL-40 promoter. Indeed, Singh et al., (2011) showed that NFI-X3 binds to NFI elements in glioma cells and it may be recruited together with STAT3. Investigation of the molecular mechanism of NFI-mediated gene activation has led to discoveries that NFIs bind to their specific binding elements, but can also interact with histone H3 (Muller and Mermod, 2000) and remodeling complexes, including Brg1 (Zhao et al., 2005). It will be important to determine if chromatin remodeling is a mechanism regulating NFI-dependent transcription of YKL-40. Singh et al., (2011) also showed that both NFI-X3 and STAT3 weakly bind to the adjacently located NFI and STAT elements on the YKL-40 promoter. Therefore, STAT3 may stabilize the weak binding of NFI-X3 and also recruit NFI-X3 to the YKL-40 promoter. Further investigation is required to determine the involvement of other transcription factors that may form complexes with STAT3 and NFI-X3. It is also important to determine if these transcription factors can form an enhanceosome with cooperative binding, like that of the IFN- β enhanceosome (Escalante et al., 2007). More exploration is also needed to

determine the subset of genes that are regulated by the STAT3 and NFI-X3 complex. In summary, we propose that NFI-X3 and STAT3 form a complex via the alpha-helical domain of NFI-X3 and this complex induces YKL-40 expression by stabilizing the weak binding of NFI-X3 to the YKL-40 promoter.

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