

ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF
SCIENCE ENGINEERING AND TECHNOLOGY

**INVESTIGATION OF THE INTERACTIONS BETWEEN NFIB AND
POTENTIAL BINDING PARTNERS MAD2B AND ATXN3**



M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

Molecular Biology-Genetics and Biotechnology Programme

Thesis Advisor: Assist. Prof. Dr. Aslı KUMBASAR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

**NFİB PROTEİNİNİN POTANSİYEL BAĞLANMA PARTNERLERİ OLAN
ATXN3 VE MAD2B İLE ETKİLEŞİMİNİN ARAŞTIRILMASI**

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To my family,



FOREWORD

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ABBREVIATIONS

NFI	: Nuclear Factor One
KO	: Knockout
CNS	: Central Nervous System
VZ	: Ventricular Zone
OL	: Oligodendrocyte
AS	: Astrocyte
BCA	: Bicinchoninic acid
DMEM	: Dulbecco's Modified Eagle Medium
DMSO	: Dimethyl sulfoxide
DNA	: Deoxyribonucleic acid
DTT	: Dithiothreitol
BSA	: Bovine serum albumin
FBS	: Fetal bovine serum
HEK	: Human embryonic kidney
LB	: Luria-Bertani broth
MH-1	: Mad homology domain-1
HCl	: Hydrochloric acid
RT	: Room temperature
SB	: Sample buffer
T_m	: Melting temperature
IP	: Immunoprecipitation
kDa	: Kilodalton
bHLH	: basic helix-loop-helix
CGN	: Cerebellar Granule Neuron
MG	: Malignant Glioma
GFAP	: Gial Fibrillary Acidic Protein
PBS	: Phosphate Buffered Saline
PKCθ	: Protein Kinase C θ
SDS-PAGE	: Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
TBS	: Tris-Buffered Saline
TBS-T	: Tris-Buffered Saline Tween-20
Amp	: Ampicillin
AD	: Activating Domain
BD	: Binding Domain
DBD	: DNA Binding Domain
Y2H	: Yeast Two-Hybrid
DUB	: Deubiquitination
UIM	: Ubiquitin Interaction Motif
JD	: Josephin Domain
UPP	: Ubiquitin-Proteasome Pathway
MAD	: Mitotic Arrest Deficient



SYMBOLS

μ	: Micro
g	: Gram
L	: Litre
m	: Mili
M	: Molar
v	: Volume
w	: Weight
μL	: Microlitre
bp	: Base pair
μg	: Microgram
h	: Hour
kDa	: Kilo dalton
min	: Minute
mM	: Milimolar
sec	: Second
V	: Volt
μM	: Micromolar



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INVESTIGATION OF THE INTERACTIONS BETWEEN NFIB AND POTENTIAL BINDING PARTNERS MAD2B AND ATXN3

ABSTRACT

Nuclear Factor I (NFI) family of transcription factors are site-specific DNA-binding proteins that play roles in viral DNA replication and regulation of gene expression. NFI proteins bind with high affinity to the dyad symmetric consensus sequence TTGGC(N5)GCCAA on duplex DNA as homo or heterodimers. Four NFI genes (*NFIA*, *NFIB*, *NFIC* and *NFIX*) have been identified in every studied vertebrate species from *Xenopus* to human. NFIs contain an N-terminal DNA binding and dimerization domain and a C-terminal transcriptional activation and repression domain. The N-terminal DNA-binding/dimerization domain (DBD) is highly conserved between the four genes and includes an MH1 motif also found in SMAD proteins of the TGF- β signaling pathway. In contrast, the proline rich C-terminal domain is less conserved. Furthermore, C-terminal domain, which encodes the transcription modulation domain, varies among the NFI family members.

NFI transcription factors are expressed in a cell-type specific fashion and may regulate tissue-specific gene expression during mammalian development. *NFIA*, *NFIB*, and *NFIX* are expressed in the ventricular zones in the developing nervous system, and in the adult neural stem cell niches. NFIs can both activate and repress transcription. Previously, a yeast two hybrid screen of a fetal human brain cDNA library using *NFIB* as bait identified *ATXN3* and *MAD2B* as potential *NFIB* binding partners.

ATXN3 belongs to the family of polyglutamine proteins which are implicated in nine different genetic neurodegenerative disorders. *ATXN3* is generally a cytoplasmic protein, widely expressed in neural and non-neural human tissues. *ATXN3* has been associated with the ubiquitin–proteasome system as well as transcriptional regulation. *MAD2B* is also known as *MAD2L2* and *Rev7* and is homologous to the spindle checkpoint gene *MAD2*. *MAD2B* is an important intermediary of the spindle assembly checkpoint. *MAD2B* is also involved in cell cycle regulation where it was shown to inhibit the Anaphase Promoting Complex (APC) by binding to Cdc20/Cdh1. *MAD2B* also acts in the DNA damage response in mammalian cells. Moreover, this protein carries the HORMA domain, responsible for protein-protein interactions.

Both proteins can regulate developmental processes by transcriptional regulation. In mice that are deficient in *MAD2B*, primordial germ cells (PGCs) die after being specified due to misregulation of epigenetic reprogramming. Deubiquitinating activity of *ATXN3* is critical for the nervous system. *ATXN3* null mice exhibit neuronal dysfunction and neurodegeneration.

In this study, in order to explore interactions between NFIB and ATXN3 or MAD2B, we performed co-immunoprecipitation experiments and GST pull down assays. To this end, epitope tagged MAD2B, ATXN3 and NFIs were overexpressed in HEK293T cells and binding was assessed via co-immunoprecipitation. We found that MAD2B and NFIB could be co-immunoprecipitated with each other. Other NFI family members (NFIA, NFIC, NFIX) did not appear to bind MAD2B. In addition, we also tested for binding of several mutant NFIB constructs that comprise only the NFIB N-terminal DNA binding and dimerization domain (NFIB_N) or C-terminal transactivation-repression domain (NFIB_C) to MAD2B. Both mutant NFIB proteins could not precipitate with MAD2B. On the other hand, ATXN3 did not co-immunoprecipitate with NFIB and NFIB did not co-immunoprecipitate with ATXN3. Furthermore, we analyzed binding of these proteins using an alternative approach, the Glutathione-S transferase (GST) pull down assay. GST pulldown experiments with NFIs and ATXN3 showed that ATXN3 pulled down NFIA and NFIB. To find out which NFIB domains are required for ATXN3 binding, we performed GST pulldown experiments using mutant NFIB proteins. Once again, these mutant constructs were NFIB_C, NFIB_N and finally, NFIB_E which encodes for NFIB DBD-engrailed repressor fusion protein. Preliminary data indicate that GST-ATXN3 precipitated NFIB_N and NFIB_E but not NFIB_C. We, therefore, surmised that NFIB may interact with ATXN3 through its N terminal domain in these experiments. Further experiments that examine these interactions *in vivo* are required to verify these results. NFIB and binding partner domains that are involved in these putative interactions, can then be dissected by site-directed mutagenesis.

In conclusion, the results obtained from this study will help elucidate the regulation of NFIB by various interaction partners, which in turn will help uncover the role of NFI family in neural development.

NFIB PROTEİNİNİN POTANSİYEL BAĞLANMA PARTNERLERİ OLAN ATXN3 VE MAD2B İLE ETKİLEŞİMİNİN ARAŞTIRILMASI

ÖZET

Nükleer Faktör I (NFI) ailesi, DNA’da spesifik bölgelere bağlanan bir protein ailesidir. Bu proteinler, viral DNA replikasyonunda ve transkripsiyonel regülasyonda rol oynarlar. NFI transkripsiyon faktörü ailesi embriyonun gelişimi boyunca ve yetişkin kök hücre nişlerinde gen regülasyonunda görev almaktadır. DNA-bağlanma analizi deneyleri ile NFI proteinlerinin DNA üzerinde çift simetrik korunmuş bir dizi bölgesine bağlandığı ortaya çıkarılmıştır. Bu çift simetrik dizi, çift zincirli DNA üzerinde TTGGC(N5)GCCAA sekansı olarak bulunmaktadır. NFI proteinleri bu korunmuş bağlanma bölgesine homo ya da heterodimer olarak yüksek afinite ile bağlanmaktadır. Dört NFI geni, NFIA, NFIB, NFIC ve NFIX, *Xenopus* türünden insan’a kadar incelenen bütün omurgalılarda tanımlanmıştır. NFI ailesinin üyesi olan tüm proteinler, N ucunda DNA bağlanma ve dimerizasyon bölgesi ve C ucunda transkripsiyonel aktivasyon ve represyon bölgesi içermektedirler. N ucunda bulunan DNA bağlanma/dimerizasyon bölgesi, NFI genleri arasında güçlü bir şekilde korunmuş bir bölgedir. Bu bölge 200-220 amino asit bir uzunluğundadır. Bu bölgede bulunan 4 adet sistein tüm NFI DNA bağlanma bölgeleri arasında korunmuştur. Ancak bu DNA bağlanma bölgesi heliks-turn-heliks gibi diğer sınıf iyi tanımlanmış DNA bağlanma motifleri ile bir benzerlik göstermemektedir. Ek olarak, bu korunmuş bölge TGF- β sinyal yolağında görevli SMAD proteininde de bulunan MH1 motifini içermektedir. Buna karşılık, prolin bakımından zengin olan C ucu bölgesi, N ucu bölgesi ile karşılaştırıldığında daha az korunmuştur. Ayrıca, NFI proteinlerinde çeşitlilikler daha çok C ucu alanında oluşmaktadır. Bu proteinler büyük olasılıkla farklı transkripsiyon modülasyon alanlarını kodluyorlar.

NFI transkripsiyon faktörlerinin gen anlatım örüntüsü hücre tipine göre değişmektedir. NFI transkripsiyon faktörleri, memeli gelişimi esnasında dokuya özel gen anlatımında önemli bir rol oynamaktadır. Özellikle, merkezi sinir sisteminde gen anlatımları ile kök hücrelerin farklılaşmasını kontrol etmektedir. NFI transkripsiyon faktörlerinin hedef genlerinin promotorlarına bağlanarak ifadelerinin aktivasyonuna ya da baskılanmasına yol açabilmektedirler. Literatürde, protein-protein etkileşimlerini keşfetmek için kullanılan maya ikili hibrit sistemi ya da Y2H yöntemi ile NFI faktörlerinin histonlar ile etkileştiği gösterilmiştir. Ayrıca bu etkileşim transfekte edilen memeli hücre deneyleri ile doğrulanmıştır. Daha sonra maya iki ikili hibrit sistemi ile bir insan fetal beyin cDNA kütüphanesi taranmıştır. Tarama esnasında NFIB yem olarak kullanılmıştır. Bu tarama sonucunda yem olarak kullanılan NFIB ile güçlü bağlanma gösteren proteinler tanımlanmıştır. Potansiyel partnerler arasından olası etkileşimlerin araştırılması için ATXN3 ve MAD2B proteinleri seçilmiştir.

ATXN3 proteini, poliglutamin protein ailesinin bir üyesidir ve bu protein ailesi dokuz farklı nörodejeneratif genetik hastalık ile ilişkilendirilmiştir. ATXN3 proteini genel olarak sitoplazmik bir proteindir. Yaygın bir biçimde nöral ve nöral olmayan insan dokularında ifade edilmektedir. ATXN3 gen anlatımının seviyesi insan beyninin farklı kısımlarında çeşitlilik göstermektedir. İnsan beyninde ATXN3 gen anlatımı, spesifik nöron alt popülasyonlarında gözlenmiştir. Ayrıca, ATXN3 proteininin ubiquitin-proteazom sistemi ilişkisi ve transkripsiyon düzenlenmesinde rol oynadığı ve etkisi gösterilmiştir.

MAD2B bir insan genidir ve MAD2L2 ve Rev7 olarak da adlandırılır. Bu protein, spindle checkpoint geni olarak adlandırılan MAD2 ile homologdur. İnsan MAD2B proteini, MAD2 (mitotic arrest deficient 2) proteini ile amino asit dizisi bakımından %53 oranında benzerlik göstermektedir. MAD2B, hücre döngüsünde spindle assembly checkpoint (SAC) olarak adlandırılan kontrol noktaları için önemli bir araçtır. MAD2B proteini birçok hücresel fonksiyonlarda da yer almaktadır. Bu fonksiyonlardan biri olan hücre döngüsü regülasyonunda, Cdc20/Cdh1 proteinlerinde bağlanarak anafaza geçiş kompleksini (APC) durdurduğu gösterilmiştir. Bu protein, memeli hücrelerde DNA hasar yanıtında da önemli bir işleve sahiptir. Ek olarak, MAD2B proteini yapısal ve fonksiyonel olarak HORMA bölgesini taşımaktadır.

ATXN3 ve MAD2B transkripsiyonel regülasyon özellikleri ile gelişimsel süreci düzenlemektedirler. ATXN3 ve MAD2B proteinlerinin yokluğunda anormal beyin gelişimi ve hücre oluşumu bozukluklarına yol açmaktadır. Ayrıca ATXN3 ve MAD2B mutant (-/-) farelerde fenotip incelendiğinde her iki proteinin de embriyonik gelişimde rol aldığı gözlenmiştir. MAD2B geninin susturulduğu farelerde, embriyolarında primordiyal eşey hücrelerinin (PGCs) farklılaştıktan sonra kaybolduğu ve epigenetik programlamanın sürdürülemediği gözlenmiştir. ATXN3 ise, deubikitinasyon aktivitesi nedeniyle sinir sisteminde önemlidir. ATXN3 mutant (-/-) farelerde yapılan çalışmalarda nöronal işlev bozuklukları ve nörodejenerasyon gözlenmiştir.

Bu çalışmada NFIB ile ATXN3 ve MAD2B etkileşimini gösterebilmek için, birlikte immünçöktürme ve GST ile çöktürme deneylerini gerçekleştirdik. Bu deneylerin sonucunda, epitop etiketli MAD2B, ATXN3 ve NFI proteinleri birlikte ifade artırımı yapıldıktan sonra proteinlerin bağlanmaları birlikte çöktürme deneyi ile analiz edilmiştir. İmmünçöktürme deneylerinin sonucunda, MAD2B ve NFIB proteinlerinin birlikte çöktüğünü gözlemledik. İki proteinin de karşılıklı olarak birbirlerini çöktürdüğünü de gösterdik. Diğer NFI proteinleri, NFIA, NFIC ve NFIX proteinlerinin MAD2B ile birlikte çökmediği yani bir etkileşimin olmadığını gözlemledik. Bu deneylere ek olarak, mutant NFIB proteinleri ile birlikte çöktürme deneyini gerçekleştirdik. Deneyde kullanılan NFIB dominant negative mutant proteinleri ya yalnızca N-ucu DNA bağlanma dimerizasyon bölgesini (NFIB_N) ya da C-ucu transkripsiyon aktivasyon ve baskılama bölgesini içermektedir (NFIB_C). Bu deneylerde mutant NFIB proteinlerinin her ikisinin de MAD2B protein ile birlikte çöktüğü gözlenmemiştir.

Diğer yandan, GST-ATXN3 füzyon proteininin ve NFI proteinleri birlikte çöktürme deneylerini gerçekleştirdik. GST çöktürme deneyleri sonucunda GST-ATXN3 proteinin hem NFIA hem de NFIB proteinini çektiği gözlenmiştir. Hangi NFIB bölgesinin bağlanmada etkili olduğunu gözlemlemek amacıyla, immünçöktürme deneyinde de kullanılan NFIB dominant negative mutant proteinlerinin GST-ATXN3 ile etkileşimi ölçülmüştür. Deney sonucunda hem NFIB_N, hem de N terminal bölge ile Engrailed represör bölgesini içeren NFIB_Eng füzyon proteinin (NFIB_E), GST etiketli NFIB protein ile birlikte çöktüğü gözlenmiştir. Sadece C ucu içeren mutant NFIB, GST-ATXN3 ile birlikte çökmemiştir. Bu nedenle, yalnızca C ucunun ATXN3 ile etkileşim için yeterli olmadığı sonucunu çıkarmaktayız.

Bu çalışmadan elde edilen sonuçlar, NFIB proteinin farklı etkileşim partnerleri tarafından regülasyonu ve dolyısıyla NFI ailesinin nöral gelişimdeki rolünü aydınlatmaya katkıda bulunacaktır.





INTRODUCTION

1.1 Nuclear Factor One (NFI) Family

Nuclear Factor I (NFI) family of animal-specific transcription factors consist of four members, NFIA, NFIB, NFIC, and NFIX, which are highly conserved from chicken to human (Gronostajski 2000). This family of transcription factors are essential for mammalian development (Whittle et al, 2009). NFI genes have not been found in unicellular organisms, which suggests that NFI genes may play key roles in complex metazoan biology (Fletcher et al.,1999). The NFI family members are mainly expressed within the central nervous system (CNS), found in the ventricular zones populated by stem cells and progenitors, as well as the lung, liver, and skeletal-muscles (Mason et al., 2011).

NFI protein was first isolated from HeLa cell from nuclear extracts and was found to stimulate initiation of adenovirus DNA replication. Indeed, NFIs are site-specific DNA-binding proteins, bind to the dyad symmetric consensus sequence TTGGC(N5)GCCAA on duplex DNA, as dimers. NFI binding motifs have been detected in promoters of genes expressed in various organs, including the brain, lung, liver, intestine, muscle, connective tissue, and skeletal elements (Gronostajski et al, 2000). NFIs can also bind individual half sites (TTGGC or GCCAA) specifically (Meisterernst et al., 1988). However, the binding affinity decreases ($K_d \sim 10^{-9}$ M) when compared to that of dyad symmetric site ($K_d \sim 10^{-11}$ M). Hence, NFIs have also been defined as TGGCA-binding proteins. NFIs can form both homo- and heterodimers. However, dimer formation depends on co-translation of proteins; they do not dimerize if they are translated separately (Sippel and Kruse, 1994). The typical NFI protein is composed of an N-terminal DNA-binding/dimerization domain (DBD) and a C-terminal transcriptional activation or repression domain (Figure 1.1). The 200-220 amino acid long N-terminal DBD is highly conserved among the four NFI genes (Figure 1.3). The DBD also contains four cysteine residues that are conserved between all NFIs. However, this domain is not similar to any of the well-characterised classes

of DNA binding domains such as the helix-turn-helix (Bandyopadhyay and Gronostasjki, 1994). Interestingly, the proline rich C-terminal domain is less conserved compared to the N-terminal domain (Figure 1.2). Moreover, considerable variation occurs within the C-terminal domains of the NFI proteins, which encodes the transcription modulation domain. Additional variation between NFI proteins is generated through differential splicing of transcripts from each of the four genes (Sippel and Kruse, 1994). NFI family members bind to same DNA sequence with similar affinity because of their highly conserved DNA binding domain. However, due to the variability in their C terminal domain, each member may have unique interactions and perform specific roles.

The only homology found between NFIs and other transcription factors is the MH1 domain located in the N-terminal region that resembles the MH1 domain of SMAD proteins (Sadreyev and Grishin, 2003). The MH1 domain of SMADs contains two subunits: one of these subunits is responsible for binding to the major groove of DNA while the other domain mediates protein-protein interactions. NFIs contain conserved Cys-Cys-His-Cys motif at the N-terminal, which is markedly similar to the zinc binding Cys-His box of the MH1 domain of SMADs (Sadreyev and Grishin, 2003).

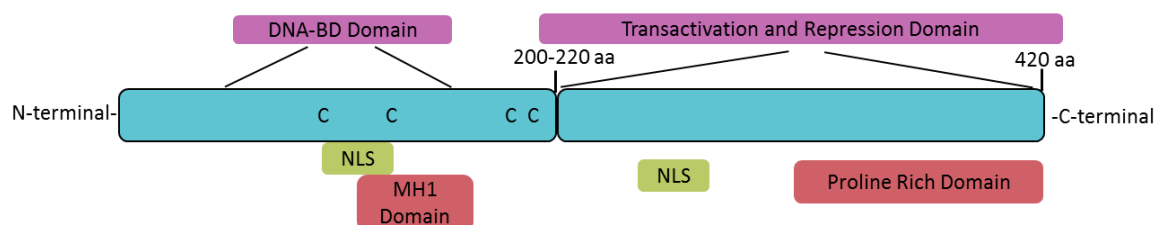


Figure 1.1: Schematic representation of NFI transcription factor. N-terminal region contain DNA-binding domain (DNA-BD) and Mad homology domain 1 (MH1).C terminal region contains transactivation and repression domain and Proline rich domain. Nuclear localization signals (NLSs) are found both region.

Of the four members that comprise the NFI family, all have numerous alternative splicing isoforms. In humans, NFIs have multiple isoforms (isoforms are available from UniProtKB with ID Q12857, O00712, P08651, Q14938). According to a recent report, a new NFIB isoform, NFIB4, lacks the DNA binding and dimerization domain. NFIB4 acts with NFIC to regulate megakaryopoiesis without heterodimerizing with NFIC or binding DNA (Chen et al., 2014).

NFI family members regulate target genes that are involved in epigenetic modification of the genome. Recently, chromatin-modifying protein Enhancer of zeste homolog 2 (EZH2) has been shown to be a target for transcriptional repression by NFIB (Piper et al., 2014). EZH2 plays an important role in promoting progenitor cell self-renewal during cortical development. *NFIB* knockout mice exhibited upregulation of EZH2 expression and increased number of proliferative ventricular zone cells within the neocortex and hippocampus. NFIB binds to the *EZH2* promoter and represses *EZH2* transcription. Moreover, in the absence of NFIB, EZH2 target genes are misregulated. Thus, one of the ways NFIB regulates cortical progenitor differentiation appears to be its role in reversing EZH2-mediated epigenetic repression.

Self-renewal and differentiation of neural progenitor cells need to be regulated for proper development of the nervous system. These two fundamental processes are linked and controlled by different signalling pathways. The Notch signalling pathway promotes neural progenitor cell self-renewal. NFIA and NFIB regulate both spinal cord and neocortical development (Deneen et al., 2006, Namiyama et al., 2009). In the cerebral cortex, NFIA activates expression of astrocyte-specific genes, while downregulating the Notch signalling pathway via repression of *HES1*, a Notch effector that maintains neural progenitor cells (Piper et al., 2010). In the spinal cord, NFIs regulate glial differentiation by controlling the expression of other Notch effectors such as *HES5*, which inhibits neurogenesis, and proteins such as GLAST, an early marker of gliogenesis (Deneen et al., 2006). NFIA induces GLAST in the ventricular zone, so that progenitors take on the glial fate. Moreover, NFIA induces the Notch effector *HES5* in the ventricular zone thereby maintaining a pool of progenitors. At the later stage, NFIA promotes terminal astrocyte differentiation. Here *Olig2* antagonizes NFIA function via direct interaction in oligodendrocyte precursors (Deneen et al., 2006).

Temporal regulation of genes are important for setting up the neural circuitry during development. NFIA regulates cerebellar granule neuron (CGN) dendritogenesis via NFI-dependent developmental switch (Ding et al., 2016). NFIA temporal binding and developmental switch programming in CGNs are controlled by an additional transcription factor. ETV1 is a member of the ETS family, enhances NFIA binding to regulate late genes in CGNs. ETV1 expression increases with maturation and correspondingly NFIA temporal occupancy of regulated late genes is increases. That

is to say, the mutual dependent interaction between NFIA and ETV1 co-regulate dendrite/synapse related genes temporal expression. Additionally NFI coordinates various differentiation processes such as axon outgrowth, dendrite formation and migration of cerebellar granule cell development via regulation of cell adhesion molecules, such as ephrin B1 and N-cadherin (Wang et al., 2007). NFI family members appear to be versatile regulators in the developing CNS, having diverse functions and targets that promote the differentiation of significant neural cell types allied to the developmental context.

1.1.1 NFI knockout (KO) studies

NFIs differ in their capabilities to either activate or repress transcription (Piper et al., 2014). Deletion of NFI genes lead to specific phenotypes for each gene, suggesting that they may have specific and significant roles in development of different organ systems, including the brain (NFIA, NFIB, NFIX) (das Neves et al., 1999; Steele-Perkins et al., 2005; Driller et al., 2007); lung (NFIB) (Steele-Perkins et al., 2005); tooth (NFIC) (Steele-Perkins et al., 2003); intestine, and skeleton (NFIX) (Driller et al., 2007).

Each NFI member plays a major role in various developmental processes in mammals. For example, deletion of *NFIA* or *NFIB* in mice causes perinatal lethality and forebrain defects. There is evidence for an important developmental role for NFI proteins in humans as well. For example, mutation in one copy of *NFIA* causes perinatal lethality, hydrocephalus, and agenesis of the corpus callosum leading to central nervous system malformation (Lu et al., 2007).

Furthermore, NFIA, NFIB, and NFIC deficient mice show the following specific defects. Mice lacking NFIB exhibit failure in lung maturation and die after the birth of respiratory failure. NFIC deficient mice display abnormal tooth development such as molars lacking roots and incisor defects (Steele-Perkins et al., 2003). The absence of NFIB leads to a significant delay in the formation of corpus callosum (Piper et al., 2009). Interestingly, NFIB appears to regulate expression of cell surface receptors (Neuropilin 1) and axon guidance molecules on both the midline glia and pioneering neurons crossing the midline which in turn may lead to the callosal dysgenesis (Piper et al., 2009). NFIA, NFIB and NFIX have all been found to regulate terminal differentiation of granule cells in the postnatal cerebellum (Wang et al., 2007, Piper et

al., 2011). However, NFIX has also been found to control development of Purkinje cells as well as Bergmann glia and velate protoplasmic astrocytes (Piper et al., 2011).

The *C. elegans* genome contains only one NFI gene, which is homologous to its mammalian counterparts only in its DBD. Nevertheless, mutants that lack NFI also display developmental abnormalities such as reduced lifespan, defects in movement and pharyngeal pumping, and delayed egg-laying (Whittle et al., 2009).

Overall, deletion of each NFI leads to similar phenotypes in the CNS (reviewed in Mason et al., 2009 and Harris et al., 2015) suggesting that these transcription factors might have similar functions. Deletion of one the NFIs results in increased expression of other NFI proteins. Thus, deficiency of one member of NFI family might be compensated by increased expression of another member (Mason et al., 2009).

Protein sequence percent homology						
Full length	61.9	66	73.2	65	60.9	61.2
N-terminus	87.4	85	89	80.7	85	80.8
C-terminus	45.5	52.1	60.4	50.5	39.2	42.9
	NFIA/NFIB	NFIA/NFIC	NFIA/NFIX	NFIB/NFIC	NFIB/NFIX	NFIC/NFIX

Figure 1.2: Percent homologies of NFI family members in mouse. Analysis was performed the aid of Clustal W. NFIA and NFIX are the most homologous, with an overall homology of ~73% (Mason et al., 2008).

1.1.2 Binding partners of NFI family

Several proteins have been found to interact with NFI family members and regulate expression of NFI target genes. Recently, Grabowska et al. (2014) looked for transcription factors that are involved in the AR/FOXA1 complex and may further regulate prostate-specific gene expression. NFIs were found to be interaction partners of FOXA1 and as part of the AR/FOXA1/NFI complex, bind to androgen response elements (AREs). Knockout studies show that silencing of NFIs in prostate cell lines led to misregulation of AR target genes (Grabowska et al., 2014).

NFIs interact with other transcription factors to regulate glial differentiation. NFIA and SOX9 form a complex that mediates the initiation of gliogenesis by regulating genes that are activated during this process (Kang et al., 2012). Moreover, NFIA binds and prevents SOX10 from inducing genes required for oligodendrocyte differentiation.

Similarly, SOX10 antagonizes NFIA function and inhibits astrocyte differentiation. Mutual inhibition by these factors was enhanced by OLIG2, a transcription factor that plays a key role in the specification of the oligodendrocyte fate. NFIA and SOX10 mutually inhibit one another to fine tune the progenitors decision to take on astrocyte versus oligodendrocyte fate (Glasgow et al., 2014).

Elsewhere, NFIs interact with other transcription factors to regulate the switch from embryonic to fetal gene expression during myogenesis (Messina et al., 2010). NFIX binds Protein kinase C θ (PKC θ) and MEF2A complex which enables and enhances PKC θ mediated phosphorylation and activation of MEF2A which in turn induces expression from MCK, a fetal muscle specific gene. NFIX gain and loss of function studies support the model that this complex induces fetal muscle specific gene expression (Messina et al., 2010). Thus, NFIs bind other transcription factors and regulatory proteins to regulate glial differentiation and myogenesis.

Another binding partner of NFI, the catalytic subunit of calcineurin weakly interacts with NFI protein and regulates its phosphorylation status. NFIs are dephosphorylated by calcineurin in malignant glioma (MG) cells, most common adult brain tumor cells (Brun et al., 2013). MG cells express glial fibrillary acidic protein (GFAP) and brain fatty acid binding protein (B-FABP), a marker of MG cells. Expression of these markers is controlled by NFIs. Dephosphorylation of NFIs by calcineurin induces expression of GFAP and B-FABP (Brun et al., 2013). Calcineurin had been shown to activate transcription domain of NFIC (Alevizopoulos et al., 1997).

A yeast two hybrid screen that searched for NFIX interacting partners identified high mobility group protein 1 (HMGN1) and CGG triplet repeats binding protein 1 (CGGBP1) (Singh et al., 2009). Indeed, in MG cells, NFIX forms a complex with HMGN1 and CGGBP1 and binds the HSF1 promoter to inhibit its expression. HSF1 (Heat Shock Factor) gets recruited to heat shock response regions and activates genes associated with heat shock response. Interestingly, HSF1 also binds to the HSF1 motif in the NFIX promoter and represses NFIX expression (Singh et al., 2009).

Crawford et al. (1998) found that NFIs play a role in controlling phosphoenolpyruvate carboxykinase (PEPCK) gene expression. PEPCK is an enzyme in the metabolic pathway of gluconeogenesis and is transcriptionally regulated. All NFIs can alter the level of expression PEPCK gene via binding the NFI motif immediately proximal to

the cAMP regulatory element (CRE) on PEPCK promoter. However, even in the absence of NFI binding motif on the PEPCK promoter, NFI could regulate its transcription. A mammalian two-hybrid system found that the C-terminal transactivation domain of NFIC can directly interact with the CREB binding domain of the CREB-binding protein (CBP). Thus, NFI can maintain the low level of transcription of the PEPCK by binding to the CREB-binding site on CBP and preventing from interacting with CREB (Leahy et al., 1999).

Crawford et al. (1998) exhibit that NFI proteins based on their ability to alter the level of basal transcription play a role in controlling phosphoenolpyruvate carboxykinase (PEPCK) gene expression. PEPCK is an enzyme in the metabolic pathway of gluconeogenesis and regulated at the transcriptional level. NFI binding sequence presents on the PEPCK gene promoter (5' of the cAMP regulatory element (CRE)). While NFIA and NFIB stimulate basal transcription, NFIC and NFIX are inhibitory in HepG2 cells (Crawford et al., 1998). All NFIs alter the level of expression PEPCK gene via binding sequence immediately adjacent to CRE on PEPCK promoter. Both CRE and NFI binding sites on PEPCK gene promoter are occupied transcription factors to alter transcription level of PEPCK gene. Even in the absence of NFI binding site on PEPCK gene promoter, NFI regulates the transcription of PEPCK gene. Therefore, NFI binds with co-activators on the promoter. The mammalian two-hybrid system demonstrates that direct interaction between the C-terminal transactivation domain of NFIC and the CREB binding domain of the CREB-binding protein (CBP). NFI competes with CREB for binding to the CREB-binding site on CBP. NFI maintains the low level of transcription of the PEPCK. Until NFI stimulated by hormones such as thyroid and glucagon. Therefore, transcription from this promoter is kept at basal levels due to this competition (Leahy et al., 1999).

Recently, NFIB transcription factor was shown to control cancer cell metastatic fate (Denny et al., 2016). NFIB might play an important role in chromatin accessibility. NFIB binds to DNA and increases chromatin accessibility at distal regulatory elements. NFIB transcription factors binding sites are highly enriched in the opening site of chromatin during metastatic progression in small cell lung cancer (SCLC) cells.

NFIB changes the chromatin accessibility and plays a role in gene expression that regulates metastatic cascade (Denny et al., 2016).

1.2 Investigation of NFIB Interaction Partners with Y2H

To identify NFIB interaction partners in the developing central nervous system (CNS), a yeast two-hybrid (Y2H) screen was previously performed using a human fetal brain cDNA library (Yilmaz et al 2013). This method is based on activation of a downstream reporter gene via the binding of a transcription factor onto an upstream activating sequence (UAS). For Y2H screening, the transcription factor is split into two separate fragments, the binding domain (BD) and the activating domain (AD). The BD is responsible for binding to the UAS and AD is responsible for transcription of the reporter gene driven by bait and prey interaction. The protein of interest is fused to the Gal4 DNA Binding Domain (DBD) and is used as bait. cDNA library of clones fused to Gal4 Activation Domain (AD) are used as prey. If interaction occurs between bait and prey protein, DBD and AD reconstitute the Gal4 transcription factor which then induces expression of the reporter gene. When NFIB-DBD fusion protein was used to screen a human fetal brain cDNA library, several candidate genes were identified as potential NFIB interaction partners (Yilmaz et al 2013). Among the genuine interactors, 12 genes were found to code for a full or a partial protein. Two of these potential NFIB interaction partners, ATXN3 and MAD2L2 (which codes for MAD2B protein), were thought to be good candidates to investigate because of their roles in developmental processes, localization in the cell during development, roles in regulation of other transcription factors and their knockout phenotypes. Knock-out studies show that MAD2B and ATXN3 may play important roles in regulation of molecular mechanisms during embryonic development and in the central nervous system (Watanabe et al., 2013, Schmitt et al., 2007) similar to NFIB (Gründer et al., 2002).

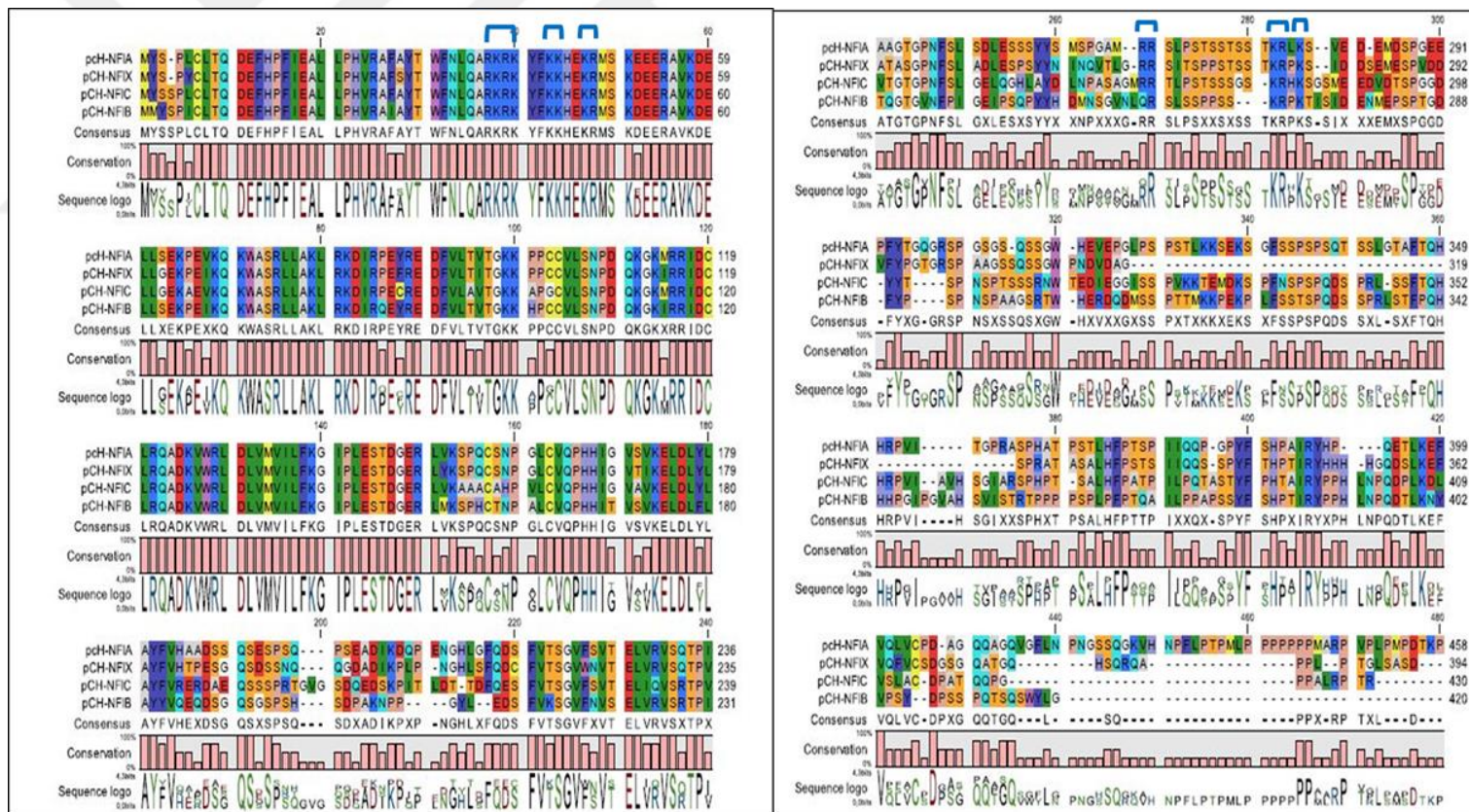


Figure 1.3: Protein sequence alignment of NFI isoforms produced in mouse. CLC Main Workbench 7 software were used to align sequences. NFIA isoform 4, NFIB isoform 2, NFIC isoform 2 and NFIX isoform 2 were used for alignment. Up to 210 amino acids represents N terminal domain, remaining part belongs to C terminal domain. Nuclear localization signals were indicated with blue indicators up to sequence.

1.3 ATXN3

ATXN3 belongs to the family of polyglutamine proteins, which are associated with nine different genetic neurodegenerative disorders. Polyglutamine diseases result from the expansion of polyglutamine (polyQ) tract in the causative proteins (Chow et al., 2004). 56 human alternative splice variants of ATXN3 have been identified so far. These variants are translated into at least 20 protein isoforms with varying predicted domain architecture (Matos et al., 2011). The longest splice variant of ATXN3 has an approximate molecular weight of 42 kDa and this variant is composed of 376 amino acids with a 22 glutamine long polyQ stretch between 296 and 317 amino acids. A shorter splice variant of ATXN3 has three ubiquitin interacting motifs (UIM) located at the C terminus. The protein sequence of human ATXN3 is highly homologous to its orthologs in rat and mouse (88%), chicken (82%) and *C. elegans* (38%), suggesting evolutionarily conserved functions for ATXN3 (Albrecht et. al, 2004).

ATXN3 protein has been identified in different eukaryotes such as protozoans, fungi, plants and animals, from nematodes to vertebrates, and is ubiquitously expressed in various tissues and cell types in humans. ATXN3 is generally a cytoplasmic protein, widely expressed in neural and non-neural human tissues. Immunohistochemical studies show that ATXN3 is localized in the cytoplasm, proximal processes, and axons and rarely in the nucleus in neurons (Trottier et al., 1998).

C-terminal domain of ATXN3 contains a polyglutamine tract and N-terminal end of the protein comprises the Josephin domain. The Josephin domain is highly conserved across a large number of different organisms ranging from the *C.elegans* to humans (Chow et al., 2014). Domain structure of ATXN3, comprises the globular N-terminal catalytic Josephin domain with deubiquitylation (DUB) activity, a polyQ sequence (Qn) and a C-terminal tail containing 2 or 3 ubiquitin interacting motifs (UIMs) between Josephin and the polyglutamine tract. Five conserved serine residues in ATXN3 UIMs are phosphorylated and one lysine residue in the Josephin domain is ubiquitylated. ATXN3 monoubiquitination increases its DUB activity and ubiquitination of the K117 of the JD, located in the vicinity of the catalytic site, is sufficient for increase in activity. ATXN3 has one functional nuclear localization signal (NLS) between the second UIM and the polyQ sequence, and it has 2 nuclear

export signals (NES) located in the Josephin domain (Matos et al., 2011). Domain structure of ATXN3 is illustrated in Figure 1.4.

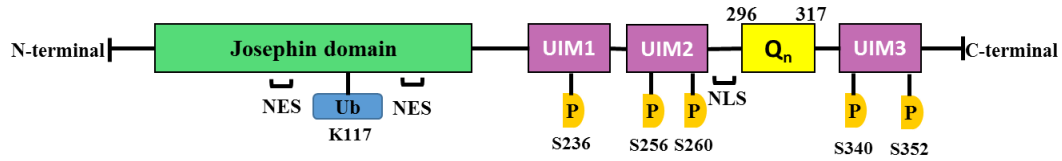


Figure 1.4: Schematic demonstration of ATXN3 gene. ATXN3 is mainly composed of a globular N-terminal catalytic domain, the JD, with DUB activity and flexible C-terminal tail containing 2 or 3 UIMS and a polyQ sequence of variable length. Five serine residues present in the UIMs (S236, S256, S260/S261, S340, S352) have been identified as phosphorylation sites, and a ubiquitinatable lysine residue was mapped to aminoacid 117, in the JD.

A recent classification defined the ATXN3/Josephin family as one of five distinct families of deubiquitylating enzymes (Week et al., 2011). The monomeric Josephin domain, which folds into a globular conformation and possesses ubiquitin protease activity, has been suggested to be the catalytic domain (Burnett et al., 2003; Chow et al., 2004). Ubiquitination is an important cellular process and has many important functions such as guiding specific proteins through endocytic pathways and targeting proteins to the proteasome, where damaged or mutated proteins are processed (Albrecht et al., 2004). The surface of the Josephin domain presents two binding sites for ubiquitin. Not only normal ATXN3 but also the expanded forms of ATXN3 are able to bind ubiquitylated proteins and the proteasome (Doss-Pepe et al., 2003). ATXN3 functions in ubiquitin-dependent pathways and itself is also regulated by ubiquitin-dependent processes. Inhibition of the catalytic activity of ATXN3 results in the increase of polyubiquitylated proteins indicating an ATXN3 involvement in ubiquitin-proteasome-pathway (UPP). Furthermore, ubiquitylation of ATXN3 is thought to regulate its enzymatic activity (Berke et al., 2005; Matos et al., 2011). ATXN3 might be auto-regulating its activity by deubiquitinating itself (Berke et al., 2005). Full length 3 UIM ATXN3 is the major isoform expressed in brain regardless of polyQ expansion level. 2-UIM containing and 3-UIM containing isoforms of ATXN3 are both considered to be “full-length” and both display similar activities, but 2-UIM ATXN3 isoform is more likely to form aggregates. Splice isoforms of ATXN3 exhibit similar enzymatic activity but show different aggregation properties (Harris et al., 2010). Three-dimensional (3D) fold of ATXN3 remains to be determined. As mentioned earlier, Josephin is a fully folded and globular domain, contrary to the C-

terminal polyglutamine-containing portion of the protein, which appears to be relatively unstructured and disordered (Masino et al., 2003).

1.3.1 Physiological role of ATXN3

Even though the physiological role and substrates of ATXN3 protein are mostly unknown, animal models and functional analyses in different cell types have shed some light on its biological functions. Rather surprisingly, two main functions ATXN3 have been assigned, one of them is that of a transcriptional repressor (Li et al., 2002; Goswami et al., 2006) and the other is that of a deubiquitylating enzyme involved in the proteasome-mediated protein degradation pathway (Donaldson et al., 2003; Mao et al., 2005).

1.3.1.1 Deubiquitinating activity of ATXN3

Several studies demonstrate that ATXN3 is involved in the ubiquitin proteasome (UPS) system and binds polyubiquitin via its ubiquitin-interacting motifs (UIMs) (Burnett et al., 2003; Chai et al., 2004). ATXN3 is a ubiquitin-specific protease (Mao et al., 2005; Nicastro et al., 2005) and is involved in the recognition of proteolytic substrates by the proteasome (Doss- Pepe et al., 2003; Berke et al., 2005). Moreover, ATXN3 regulates aggresome formation (Burnett et al., 2003) and suppresses polyQ-induced toxicity by its ubiquitin-dependent activities.

Josephin domain is the catalytic domain that displays protease activity. Both the full-length ATXN3 and the isolated Josephin domain are capable of cleaving ubiquitin substrate (Chow et al., 2005). The UIMs may be recruiting the polyubiquitinated substrates and positioning the ubiquitin chain close to the catalytic cysteine residue (Cys14) of Josephin domain, where catalytic cleavage occurs (Mao et al., 2005; Berke et al., 2005).

ATXN3 has been shown to interact with the ubiquitin, Rad23 and proteasome binding factors p97/valosin-containing protein (VCP/p97). ATXN3 interacts with VCP/p97 through C-terminal region and this interaction may be important for the recognition of ubiquitylated substrates by the proteasome. VCP is an enzyme that is a member of ATPase family, is encoded by the VCP gene in humans. Moreover, the N-terminal Josephin domain of ATXN3 has been shown to interact with ubiquitin like domains of the human homologs of yeast DNA repair protein Rad23, HHR23A, and HHR23B through the ubiquitin-binding site 2 of the JD. Rad23 is a protein that may translocate

proteolytic substrates to the proteasome (Matos et al, 2011; Doss-Pepe et al., 2003). HHR23A and B are proteasome binding factors that are involved in translocation of proteolytic substrates to the proteasome (Nicastro et al., 2009). VCP/p97 and HHR23A and B have been found to participate in shuttling of polyubiquitylated substrates to the proteasome for degradation, specifically in the endoplasmic reticulum associated degradation (ERAD) system (Matos et al., 2011). Physical interaction between ATXN3 and Rad23 as well as ATXN3 and VCP, suggests that ATXN3 has a role in the proteolytic system (Doss-PePe et al., 2003).

Recently, neural precursor cell expressed developmentally downregulated 8 (NEDD8), a ubiquitin-like protein, has been reported to accumulate in the inclusions of MJD brains. Ferro et al. (2007) showed that ATXN3 is able to directly bind NEDD8, through its Josephin domain. NEDD8 interacts with ATXN3 in a substrate-like manner, independent of the presence of UIM domains and the polyQ tract. NEDD8 is essential for the activity of SCF-like ubiquitin ligase complexes, which control degradation of several substrates and modulate the pathways where these substrate participate. Interestingly, ATXN3, the *Caenorhabditis elegans* homologue of ATXN3 also interacts with NEDD8 suggesting that this interaction is evolutionarily conserved (Ferro et al., 2007).

1.3.1.2 Transcriptional regulation by ATXN3

ATXN3 plays a role as a transcriptional regulator through various molecular mechanisms (Li et al., 2002, Evert et al., 2006, and Mason et al., 2011). Polyglutamine proteins regulate histone acetyltransferase activity (HAT) and these polyglutamine proteins are proposed to play an important role in transcription. ATXN3 acts as a transcriptional repressor of major transcriptional coactivators, CBP, p300, and p300/CBP-associated factor (PCAF). Endogenous and pathological forms of ATXN3 both interact with transcriptional coactivator proteins CBP, p300, and PCAF, through the ATXN3 C-terminal polyglutamine containing domain, and repress their transcriptional activity. On the other hand, N-termini of both normal and pathological ATXN3 bind to histones and inhibit their acetylation by blocking the access of HATs to acetylation sites (Li et al., 2002). To sum up, ATXN3 interacts with three transcriptional coactivator proteins (CBP, p300, and PCAF), two transcriptional repressor proteins (HDAC3 and NCor), and histones indicating a role for ATXN3 in transcriptional regulation (Matos et al, 2011).

Histones are thought to be excellent candidates for interacting with N-terminal Josephine domain of ATXN3, due to favorable charge complementarity between histones and the surface of the hairpin structure of Josephine domain (Nicastro et al, 2005). To regulate transcription of MMP-2, ATXN3 binds to specific chromatin regions of the gene promoter and represses transcription by recruitment of the histone deacetylase 3 (HDAC3), the nuclear receptor corepressor (NCoR), and deacetylation of histones bound to the promoter. ATXN3 binds to the promoter of the gene of interest to represses transcription by recruiting corepressors, Histone Deacetylase 3, the nuclear receptor corepressor (NCoR) and promoting histone deacetylation. Both normal and pathological ATXN3 interacts with endogenous HDAC and NCoR in cell and human brain extracts. However, normal ATXN3 increases their deacetylase activities. On the other hand, pathological ATXN3 has been found to be associated with reduced deacetylase activity and therefore, to lack its transcriptional repressor function (Evert et al., 2006).

Even though these findings clearly point to a role for ATXN3 in transcriptional regulation, it is unclear which molecular pathways are influenced by ATXN3. Arouja et al. (2011) found the forkhead box class O (FOXO) transcription factor 4 (FOXO4) to specifically interact with ATXN3. FOXO family transcription factors are involved in various cellular processes such as regulation of cell cycle arrest, differentiation, cell death and resistance to cellular oxidative stress. Arouja et al. (2011) showed that ATXN3 is a transcriptional coactivator of the manganese superoxide dismutase 2 (SOD2) gene within the FOXO-mediated oxidative stress response. In response to oxidative stress, ATXN3 and FOXO4 translocate to the nucleus, concomitantly bind to the SOD2 gene promoter and activate the FOXO4-dependent transcription of the SOD2 gene (Araujo et al., 2011).

In addition to these findings, cytoplasmic interactors of the ATXN3 protein were explored by pull-down experiments. This experiment showed that both full-length and short variants (JD alone) of ATXN3 could bind to both α - and β -tubulin isolated from rat brain extracts (Mazzucchelli et al., 2009). Moreover, ATXN3 affects the expression of several proteins related to cell structure/motility. ATXN3 regulates the degradation of integrin subunits such as α 5 integrin subunit (ITGA5), a molecular partner of ATXN3. Integrins are transmembrane cell surface receptors and are involved in many stages of neuronal development and function, such as proliferation, survival, adhesion,

cytoskeletal organization, process outgrowth and synaptic function (do Carmo Costa et al, 2010). Neves-Carvalho et al. (2015), show that ATXN3 is required for neuronal differentiation and for normal cell morphology, cytoskeletal organization, proliferation and survival of SH-SY5Y and PC12 cells. In the case of ATXN3 depletion, ITGA5 levels were deregulated through increased proteasomal degradation that leads to decreased cell adhesion and disorganization of the neuronal cytoskeleton. An expanded polyQ tract of ATXN3 or mutant versions of ATXN3 that lack catalytic activity leads to loss of ATXN3 cellular function, which in turn may be involved in molecular mechanisms underlying neurodegeneration. Abnormal morphology and reduced branching were both observed in cultured neurons where ATXN3 was knocked down by shRNA (Neves-Carvalho et al., 2015).

Thus, the present understanding of the physiological roles of ATXN3 strongly suggests that it can be regarded as a multifunctional protein, and might be involved in integrating network of protein–protein interactions (Cusick et al., 2005; Jeffery, 2004).

1.3.2 The role of ATXN3 in the nervous system

ATXN3 is a disease protein in spinocerebellar ataxia type 3 (SCA3), also called Machado-Joseph disease (MJD). SCA3 is an autosomal-dominantly inherited neurodegenerative disease caused by expansion of the CAG trinucleotide repeat (55–84 repeats) in the coding regions of the *ATXN3* gene. Furthermore, ATXN3 has been associated with the ubiquitin–proteasome system and transcriptional regulation, known physiological roles of this protein. ATXN3's actions in ubiquitin–proteasome pathway and transcriptional regulation are necessary for proper mammalian brain function (Todi et al., 2011; Tsai et al., 2004).

Modulation of gene expression is essential for mammalian brain function and chromatin remodeling is an important means of regulating gene expression. Acetylation and deacetylation of histones by histone acetyl transferases (HATs) and histone deacetylases (HDACs) respectively at lysine residues are most known and studied post-translational modifications in cognition and neuropsychiatric diseases (Volmar et al., 2005). Josephin domain of ATXN3 can interact with histones and binds specific sites on chromatin and represses transcription by recruitment of HDAC (Evert et al., 2006). Moreover, ATXN3 acts as a transcriptional repressor by inhibiting HAT

activity of major transcriptional coactivators (Li et al., 2002). These actions render ATXN3 as a significant transcriptional regulator.

In addition to its transcriptional regulatory function, the deubiquitinating activity of ATXN3 is also important for nervous system. Several molecular pathways are regulated by the post-translational conjugation of ubiquitin to target proteins. Ubiquitination or deubiquitination are both equally critical for neuronal function. Deubiquitinating enzymes (DUBs) are involved in regulation of neuronal fate determination, axonal path finding and synaptic communication and plasticity by reversing and thus modulating protein ubiquitination (Todi et al, 2011). Nervous system function is regulated by numerous ubiquitin dependent pathways involving specific DUBs and, ubiquitin dependent protein quality control might be especially vital to neuronal homeostasis. Mutations in DUBs lead to neuronal dysfunction and neurodegeneration. ATXN3 is one of these DUBs (Todi et al, 2011).

Interestingly, ATXN3 has a neuroprotective role in *Drosophila melanogaster* as it represses degeneration caused by several polyQ disease proteins, including SCA3, (Warrick et al., 2005). As described earlier, normal human ATXN3 is a polyubiquitin binding protein with ubiquitin protease activity and a suppressor of polyglutamine neurodegeneration *in vivo*. Suppressor activity of ATXN3 requires ubiquitin-associated activities and is dependent upon proteasome function. Normal ATXN3 activity mitigates the polyQ-induced neurodegeneration and potential therapeutic role of ATXN3 activity for polyglutamine disorders in this model organism (Warrick et al., 2005). Thus, ATXN3 can act in both neurodegenerative and neuroprotective processes.

A recent report presents evidence that ubiquitination of this DUB is critical for its neuroprotective role *in vivo*. Regulation of the activity of DUBs is important for homeostasis, including their own regulation. Ubiquitination regulates the neuroprotective function of ATXN3 *in vivo* (Tsou et al. 2013). Indeed, mutating the ubiquitination site at lysine 117 decreases its neuroprotective function *in vivo* (Tsou et al., 2013). Thus, ATXN3 activity is involved in both neurodegenerative and neuroprotective processes.

1.3.3 ATXN3 knock-out model

As mentioned above, ATXN3 carries out two main functions: as ubiquitin protease and as a transcriptional corepressor. To illuminate ATXN3 function, ATXN3 knock-out (KO) mice have been generated. The mostly known physiological ATXN3 result is that contribution to the pathology in SCA3, is a neurodegenerative disease caused by expansion of a polyglutamine domain in the ATXN3. To find out ATXN3 function and to test the idea that loss of ATXN3 plays a role at the pathology in SCA3, Schmitt et al. (2007) generated ATXN3 KO mice. They found that inactivation of ATXN3 in mouse increases ubiquitinated proteins in ATXN3 KO tissues. They performed Ub immunohistochemistry studies to compare mutant mice and controls ATXN3 Ub protease activity. Ub staining intensity increased in ATXN3 null mouse brains. Finally, ATXN3 KO mice studies found no evidence that supports the hypothesis that loss of physiological ATXN3 contributes to the pathology in SCA3 but they confirmed the importance of ATXN3 as a deubiquitinating enzyme *in vivo* (Schmitt et al., 2007).

Mutant ATXN3 has a toxic function and cause pathogenesis of Spinocerebellar ataxia 3 (SCA3). However, the exact mechanism of the disease remains elusive. To overcome this problem, Switonski et al (2011) create knock-in humanized mouse model. To generate the human ATXN3 knock-in model, human cDNA for ATXN3 that contains 69 CAG repeats was cloned from a SCA3 patient. Then, human ATXN3 was introduced into the mouse ATXN3 exon 2 loci while deleting mouse ATXN3 along with exon 3 and intron 2. After the human transgene was inserted into mouse ATXN3, deleted along with exon 3 and intron 2, any expression of mouse ATXN3 was abolished and the resulting mice acquired the knock-out properties. Next, RNA expression was analyzed and different splicing isoforms of ATXN3 composed of human and mouse exons were detected. Correct splicing form that contains correct exon 1/exon 2 isoform was detected via bioinformatic analyses. When they compared to this form with native mouse locus, they did not observe any change structurally and power of the splicing. As a result, they created a functional ATXN3 knock-out mouse model that is viable and fertile and does not present a reduced life span (Switonski et al., 2011).

1.4 MAD2B

MAD2B gene, also known as MAD2L2 and Rev7, is homologous to the spindle checkpoint gene MAD2 (MAD2L1). MAD (mitotic arrest deficient)-family genes MAD1, MAD2, and MAD3 were initially identified in screens for mitotic checkpoint defects. Orthologs of MADs have been characterized in a number of organisms including humans. Human MAD2B acquired its name due to its 23% sequence identity and 54% similarity to mitotic arrest-deficient 2 (MAD2) at the amino acid level. MAD2B was mapped to 1p36 and is thus distinctive from MAD2 which was mapped to 4q27 (Cahill et al., 1999; Murakumo et al., 2000). In yeast, MAD2 is required for the mitotic arrest if the spindle assembly is perturbed. The human homolog of MAD2 has been shown to be a necessary participant of the mitotic checkpoint in HeLa cells (Li et al., 1996). Human MAD2 was localized at the kinetochore after chromosome condensation. However, MAD2B was no longer observed at the kinetochore in metaphase, suggesting that MAD2B might monitor the integrity of the spindle-kinetochore attachment. Moreover, human breast tumor cell lines which exhibited reduced MAD2B expression failed to arrest in mitosis. Thus, MAD2B is a key factor in the regulation of the mitotic checkpoint (Li et al., 1996).

Both MAD2 and MAD2B structures contain a HORMA domain (Aravind and Koonin, 1998). The HORMA domain contains several conserved motifs: two amino acid residues - an arginine residue in the N-terminal region of the domain and a glutamate residue in the center of the domain. The HORMA domain is involved in protein-protein interactions, protein oligomerization, and chromatin state recognition. HORMA domain is a multifunctional protein-protein interaction module located in different eukaryotic signaling pathways including the spindle assembly checkpoint, DNA recombination/repair pathways and the initiation of autophagy (Hanafusa et al., 2015). HORMA domain proteins utilize a “safety belt” peptide interaction mechanism. This mechanism controls assembly and disassembly of signaling complexes (Rosenberg et al., 2015) (Figure 1.5).

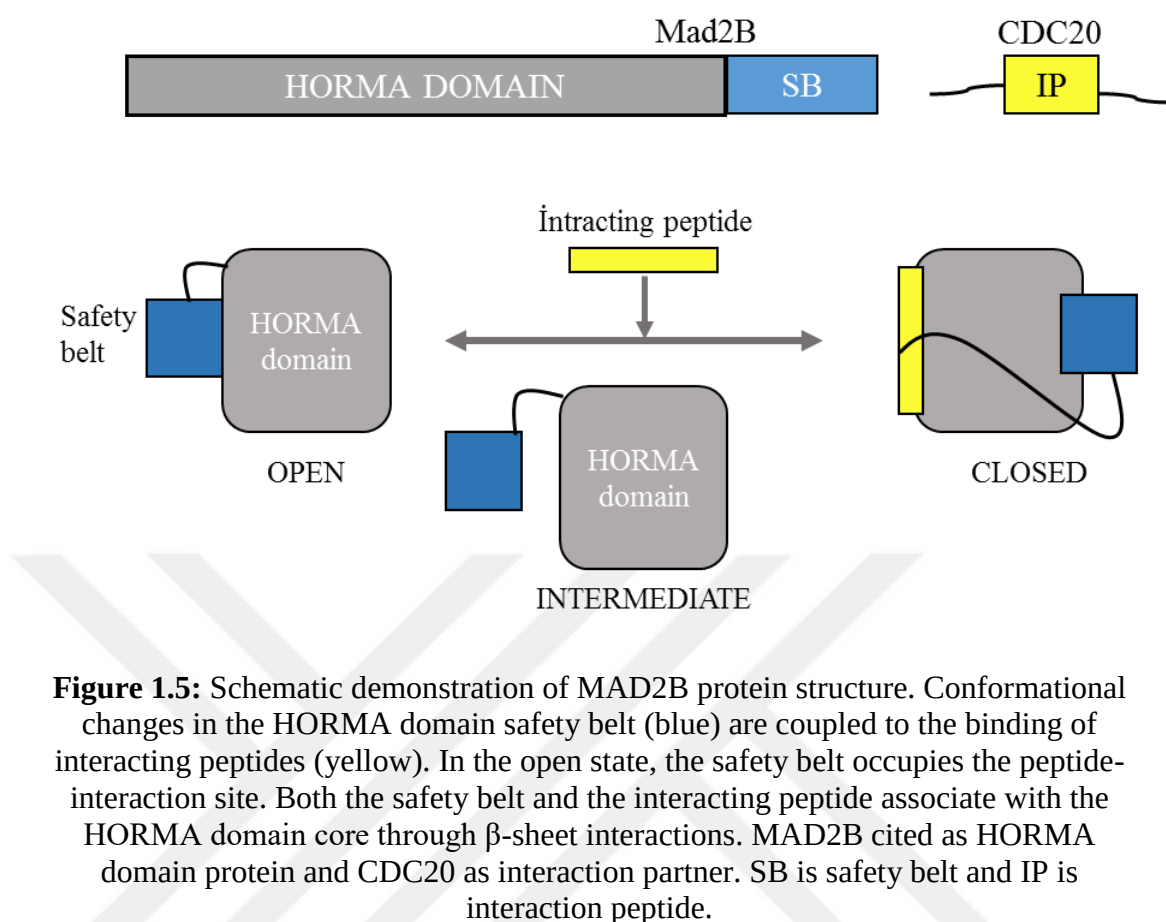


Figure 1.5: Schematic demonstration of MAD2B protein structure. Conformational changes in the HORMA domain safety belt (blue) are coupled to the binding of interacting peptides (yellow). In the open state, the safety belt occupies the peptide-interaction site. Both the safety belt and the interacting peptide associate with the HORMA domain core through β -sheet interactions. MAD2B cited as HORMA domain protein and CDC20 as interaction partner. SB is safety belt and IP is interaction peptide.

1.4.1 MAD2B regulates cell cycle progression

Proteolysis is an important process for critical cellular events of the cell cycle, in signal transduction, in transcription, and in development. During mitosis, anaphase promoting complex (APC), a ubiquitin ligase, controls both separation of sister chromatids and exit from mitosis. APC activity is essential for ordered progression of the cell cycle. APC is controlled by a network of regulatory factors: MAD2, CDC20, and CDH1. The checkpoint protein MAD2 is an inhibitor of APC, whereas CDC20 and CDH1 are activators. Just like MAD2, MAD2B is an inhibitor of APC and prevents activation of APC by CDH1 and CDC20. Therefore, MAD2B is referred to as an inhibitor of the anaphase-promoting complex (Chen and Fang, 2001). However, since MAD2B is localized in the nucleus and binds the kinetochore throughout the cell cycle, MAD2B is likely involved in cellular processes other than spindle assembly checkpoint control.

Many types of cancer cells have defects in their cell cycle machinery (Wassmann et al., 2001). Overexpression of papillary renal cell carcinoma (RCC)-associated (PRCC)

disrupts the mitotic checkpoint in human embryonic kidney cells (Weterman et al., 2001). PRCC binds to MAD2B. In cancer cells, the product of a translocation event, PRCC-TFE3 fusion protein does retain its MAD2B interaction domain, but binds weakly to MAD2B. In addition, PRCC positive tumor cells are defective in their mitotic checkpoint and overexpression of PRCC-TFE3 disrupts the mitotic checkpoint as well (Weterman, et al., 2001).

To examine the potential role that MAD2B may play in cell cycle control, Medendorp et al. (2009) performed a protein-protein interaction screen using MAD2B as a bait. They found that small GTPase RAN, a known cell cycle regulator to be a novel MAD2B interacting protein. RAN has been implicated in the control of DNA synthesis and cell cycle progression and is also involved in mitosis. Yeast two-hybrid analysis of binding between full length RAN to MAD2B deletion constructs, showed that full length MAD2B is required to bind RAN. Deletion of the HORMA domain results in abrogation of the interaction. Previously MAD2B and PRCC interaction had been shown by Weterman et al (2001). To evaluate whether PRCC might act as an integral component of the MAD2B-RAN complex, U2OS cells were transiently cotransfected with MAD2B-CFP, PRCC-dsRed and/or RAN-YFP expression constructs. Triple MAD2B, PRCC and RAN transfectants showed a partial co-localization, suggest that interactions of MAD2B with either RAN or PRCC may be dynamic in nature (Medendorp et al., 2009). Based on the same approach, MAD2B was used as a bait once again and they identified the clathrin light chain CLTA as a novel genuine interactor. Clathrin is a protein that stabilizes mitotic microtubules by forming a trimeric structure, is composed of heavy (CLTC) and light (CLTA/CLTB) chains, respectively. During mitosis, CLTA was located at the mitotic spindle, co-localizing with α -tubulin, and MAD2B exhibited a very similar localization. Knockdown of MAD2B lead to an increase in misaligned chromosomes and a redistribution of CLTA during mitosis leads to cell cycle regulation defects (Medendorp et al., 2010).

Many protein-protein interactions affect the phases of the cell cycle, some of them influence it in a negative fashion. Bacterial pathogen, *Shigella* effector IpaB causes cell-cycle arrest by targeting MAD2B. When IpaB interacts with APC inhibitor MAD2B, APC undergoes aberrant activation. IpaB-MAD2B dependent cell cycle arrest allows bacterial colonization. Thus, *Shigella* affects epithelial cell-cycle progression by delivering IpaB into host cells. IpaB, targets epithelial cells and directly

modulates their cell-cycle progression by binding of MAD2B (Iwai et al., 2007). Similar to these findings, some interaction partners of MAD2B might play a novel role in cell cycle regulation. HCCA2 (hepatocellular carcinoma-associated gene 2) was initially identified as an HCC-specific protein and overexpression of HCCA2 causes G0/G1 arrest which in turn inhibits cell proliferation (Li et al., 2007). Interestingly, MAD2B was identified as a possible interaction partner in a screen performed by this group.

In short, MAD2B is a key regulator for the cell cycle. MAD2B ensures accurate mitosis by preventing premature activation of APC. Depletion of MAD2B causes dysregulation of APC substrate degradation and unscheduled mitotic exit (Listovsky et al., 2013).

1.4.2 MAD2B controls DNA repair activities

As explained above, MAD2B acts in several cell processes including cell cycle regulation where it was shown to inhibit the Anaphase Promoting Complex (APC) by binding to Cdc20/Cdh1. MAD2B may also function as a mitotic checkpoint protein as its homolog, human mitotic checkpoint protein MAD2, performs this function. However, some studies have shown that MAD2B plays an important role in DNA damage response in mammalian cells. MAD2B binds to Cdc20 following DNA damage and binding of Cdc20 to the MAD2B-APC complex following DNA damage may alter APC substrate specificity. Murakumo et al. (2000) show that MAD2B interacts with human REV3. MAD2B and REV3 interaction domains were determined and this interaction was found to be involved in the formation of DNA polymerase ζ complex, an error-prone DNA polymerase in translesion DNA synthesis (TLS) mechanism. Genomic DNA is constantly damaged by various factors. To avoid replication arrest, cells employ a strategy termed DNA damage tolerance. One of these strategies is translesion DNA synthesis (TLS), a mechanism that bypasses replication forks blocked by lesions. MAD2B might be an adapter between DNA repair machinery and the spindle assembly checkpoint system (Murakumo et al., 2000). MAD2B may be involved in the cell cycle response to DNA damage (Murakumo et al., 2002).

Recently, MAD2B has been shown in polymerase switching mechanism. During TLS, damaged DNA is synthesized by specialized error-prone DNA polymerases such as REV1 and Pol ζ which consists of MAD2B and REV3. REV1- Pol ζ complex forms a

ternary complex and might facilitate the switch from an inserter polymerase (REV1) to the extender polymerase (REV3). Thus, REV1 binding site could provide new interface that where the polymerase switch occurs, potentially altering the DNA damage response (Kikuchi et al., 2012).

DNA repair and inhibition of DNA repair activities at telomeres are crucial to prevent genomic instability. Cancer and aging both contribute to genomic instability. MAD2B controls DNA repair activities at mammalian telomeres. MAD2B accumulates at uncapped telomeres and promotes non-homologous end-joining (NHEJ)-mediated fusion of deprotected chromosome ends resulting in genomic instability (Boesarma et al., 2015). MAD2B knockdown causes elongated 3'telomeric region overhangs suggesting that MAD2B inhibits 5' end resection (5' ends of the break are cut away in a process). Thus, MAD2B is a critical player in regulation of DNA repair activity which promotes NHEJ by inhibiting 5' end resection (Boersma et al., 2015).

1.4.3 Binding partners of MAD2B

Adenovirus death protein (ADP), the human subgroup of C adenovirus (Ad) protein, is synthesized at very late stages of infection and effect cell cycle of the host. These type of proteins function to deregulate the cell cycle, induce and repress a variety of cellular genes. A yeast two-hybrid screen that used ADP as bait, identified MAD2B (Ying and Wold 2003). ADP was found to strongly bind human MAD2B *in vitro* or *in vivo*. Cells overexpressing MAD2B, when infected with adenovirus, were lysed much more slowly compared to untransfected cells suggesting that the expression of MAD2B counteracts the cell lysis-promoting function of ADP. Another interaction partner of MAD2B is Trichosanthin (TCS), an active component of the medicinal herb used for clinical trials such as treatment of AIDS and cancer. TCS is a single chain polypeptide that inactivates the ribosome and inhibits protein synthesis by depurinating a single adenine base at 4324 (Chan et al., 2001). MAD2B interacts with TCS.

The mitogen-activated protein (MAP) kinase pathways are signaling cascades that are utilized by eukaryotic cells to respond to extracellular signals. ETS-domain transcription factor Elk-1 is one of the best studied targets of these pathways. Zhang et al. (2007) found that MAD2B is an interaction partner of Elk-1. MAD2B promotes phosphorylation of Elk-1 by the c-Jun N-terminal protein kinase (JNK) MAP kinases.

Phosphorylation of Elk-1 enhances the activity of its transcriptional activation domain. Thus, MAD2B causes upregulation of Elk-1 target genes resulting in exposure of cells to stress conditions caused by DNA-damaging agents. This study underlined an additional important role for MAD2B in the regulation of gene expression (Zhang et al., 2007).

Another interaction partner of MAD2B is T cell factor 4 (TCF4), which normally binds to β -catenin in the WNT signaling pathway and trans-activates downstream target genes such as c-Myc (oncogenic transcription factor) and cyclin D, which are involved in the development of cancer. WNT signaling pathway is critical in the regulation of cell growth and a key event of this pathway is activation of TCF4. A yeast two-hybrid screen identified MAD2B as a TCF4 binding protein. Regions of TCF4 that interact with MAD2B were identified by TCF4 deletion mapping: MAD2B was found to bind the DNA binding domain of TCF4. Moreover, silencing MAD2B enhanced the ability of TCF4 to bind the promoter of Slug, a gene involved in epithelial-mesenchymal transition (EMT). Moreover, MAD2B inhibited transcriptional activation by and DNA binding ability of TCF4. Hong et al (2009) proposed that MAD2B functions as a negative modulator of TCF4-induced EMT in colorectal cells in culture.

Taken together, reported binding partners of MAD2B include Cdh1 and Cdc20 which are substrate binding proteins of the APC/C complex, Rev1 and Rev3 (two translesion polymerases), the transcription factors Elk-1 and TCF4, the clathrin light chain A as summarized above.

1.4.4 Effect of MAD2B on the nervous system and carcinogenesis

Recent studies using different organisms such as yeast, vertebrates, and mammalian cells have indicated that aberrant expression of MAD2B may increase spontaneous and DNA damage-triggered mutations.

MAD2B is an enzyme involved in translesion DNA synthesis and has been implicated in the development of several types of cancer since genetic instability is a key factor in this process. Malignant glioma is the most common and lethal brain tumor associated with genomic alterations. As mentioned above, MAD2B encodes the regulatory subunit of Pol ζ . However, its effect on human glioma has not been identified. Zhao et al. (2011) examined the expression level of MAD2B in human glioma and normal brain tissue. They found that MAD2B was overexpressed in glioma

compared to normal brain tissue. Moreover, knockdown of MAD2B significantly reduced survival of glioma cells and enhanced apoptosis in response to ionizing radiation. These findings reveal that expression of MAD2B is deregulated in glioma and MAD2B might be a potential target for improving the efficacy of radiotherapy (Zhao et al., 2011).

Defects in mitotic spindle checkpoint genes have long been shown to cause aneuploidy in human cancer (Li et al, 1996). Many colorectal cancer cells are aneuploid and characterized by chromosomal instability. Genetic alterations cause this instability which in turn might accelerate tumorigenesis. As mentioned above, MAD2B is a key constituent of a cell cycle checkpoint mechanism that delays anaphase until all chromosomes are correctly oriented. A defect in this control mechanism contributes to genetic instability (Li et al., 1996). On the other hand, mutations in these control mechanism genes in cancer are rare, suggesting that loss-of-function mutations in the spindle checkpoint genes are not related to aneuploidy (Rimkus et al 2006). An alternative mechanism would involve misregulation of MAD2B expression which then would be a key factor in colon cancer. Additionally, significant overexpression of MAD2B in a subset of human colorectal tumors, was associated with reduced survival. The cellular function of MAD2B may be contributing to tumorigenesis, likely enhances tumor progression by the development of aneuploid cells with an aggressive phenotype. However, MAD2B was identified as a prognostic parameter, as patients with MAD2B overexpression had significantly reduced survival (Rimkus et al., 2006).

Aberrant MAD2B's may affect cancer cells treated with a chemotherapy agent. A recent study evaluated the expression of MAD2B in epithelial ovarian cancer (EOC) and showed the association between its expression and chemosensitivity in cancer cells (Niimi et al., 2014). Ovarian cancer is the fifth common malignancy and most common cause of cancer related death in women. Chemotherapeutic agents induce DNA damage in cancer cells where the DNA repair system may affect efficacy of these agents for therapy. Deregulation of DNA repair proteins cause defects in DNA repair and subsequent genetic instability and cell death. As described above, translesion DNA synthesis (TLS) is a DNA damage tolerance mechanism where MAD2B acts. Thus, targeting DNA repair proteins such as MAD2B might improve the efficacy of chemotherapeutic agents. Here, knockdown of MAD2B in clear cell carcinoma (CCC) cells decreased cell proliferation *in vitro* and *in vivo* without affecting the cell cycle.

Apoptotic cells and increased DNA damage were observed after chemotherapeutic agent treatment. To sum up, depletion of MAD2B enhances the sensitivity of CCC cells against to chemotherapeutic agents (Niimi et al., 2014).

In the CNS, MAD2B might mediate proteins which are important for development. Recently, using human fetal brain cDNA yeast two hybrid screen and co-immunoprecipitation experiments, MAD2B has been shown to bind Single-minded homolog 2 (Sim2), a transcription factor, which in humans, plays a major role in the development of the central nervous system as well as the construction of the face and head (Meng et al., 2012). MAD2B was found to be mainly expressed in cortex and hippocampus in neurons, which suggests that MAD2B may play an important role in cortical neurons. Moreover, MAD2B interacts with clathrin light chain A (CLTA) in neurons. CLTA's well-known role is in endocytosis. Overexpression of both MAD2B and Sim 2 and interaction between Sim2 and MAD2B may affect clathrin-mediated endocytosis (Meng et al., 2012).

Recently, Meng et al (2014) have represented a novel mechanism of Diabetic encephalopathy, a disease associated with brain damage caused by diabetes. Brain function changes in diabetic patients. As a result of hyperglycemia, the expression level of MAD2B increased which resulted in cyclin B1 accumulation along with deficiency in neurons or neuronal apoptosis. When MAD2B is knocked down in neurons, cyclin B1 expression was reversed, hyperglycaemia-induced apoptosis was significantly reduced (Meng et. al., 2014).

1.4.5 MAD2B involved in developmental process

As described above, MAD2B functions in diverse processes such as DNA repair, cell cycle control and regulation of gene expression. However, biological importance of these reported activities is unclear as functional analysis experiments still need to be performed.

During embryonic development, MAD2B deficient mouse primordial germ cells (PGCs) die after their specification due to defects in epigenetic reprogramming (Pirouz et al., 2013). Investigation of MAD2B's role in epigenetic reprogramming of PGCs by gain and loss of function analysis showed that MAD2B interacts with the histone methyltransferases G9a and GLP, leading to downregulation of H3K9me2. Moreover, MAD2B binds to and inhibits Cyclin dependent kinase 1 (Cdk1), which then leads to

cell cycle arrest in the G2 phase and increases in the epigenetic mark H3K27me3, via another histone methyltransferase, EZH2. Thus, MAD2B controls epigenetic transition of PGCs from H3K9me2 state at E7.5 to H3K27me3 state at E9.5 and PGC arrest in G2. In the absence of MAD2B, PGCs fail to regulate these processes (Pirouz et al., 2013).

Another example for a developmental role of MAD2B is stabilization of pluripotency in pluripotent embryonic stem cells (ESCs). Pluripotency is a transient state between totipotency and lineage differentiation in the early embryo. Pluripotent cells are derived from inner cell mass (ICM) of the blastocyst, cultured *in vitro* as embryonic stem cells (ESCs). As described above, MAD2B play key roles in development of PGCs (Pirouz et al., 2013). MAD2B is also required by pluripotent ESCs (Pirouz et al., 2015). MAD2B protein is highly expressed in mouse ESCs and its expression level decreases during embryoid body differentiation. MAD2B deficient ESCs were unstable and differentiated into primitive endoderm. Also, epigenetic marks were altered, possibly due to deletion of MAD2B. Previously, MAD2B was found to affect both FGF and WNT signaling via functional binding to the transcription factors Elk-1 and TCF-4 (Zang et al., 2007; Hong et al., 2009). In MAD2B deficient ESCs, expression of several MAPK cascade components were misregulated leading to loss of ESC pluripotency (Pirouz et al., 2015).

1.5 Aim of the Study

ATXN3 and MAD2B have been shown to be important effectors of developmental processes, such as cell cycle control, regulation of gene expression and modulation of epigenetic marks. Both proteins are expressed throughout the human brain during development and in the adult (Meng et al., 2012; Matos et al., 2011) where NFIs are also expressed. Knockout of ATXN3 leads to no gross phenotypes but silencing the gene *in vitro* interferes with neuronal differentiation (Tsou et al., 2013; Neves-Carvalho 2015). MAD2B deficient mice display defects in PGC development while *MAD2B*^{-/-} embryonic stem cells are restricted in cell fate (Hong et al., 2009, Meng et al., 2012). Moreover, ATXN3 and MAD2B both interact with and regulate transcription factors (Araujo et al., 2011; Zhang et al., 2007). Previously, ATXN3 and MAD2B have been identified as potential NFIB binding partners in a yeast two hybrid assay. Based on their cellular localization in the nervous system, their potential ability

to regulate gene expression and role in development, ATXN3 and MAD2B were chosen for further study since they were likely to interact with NFI family of transcription factors which play important roles in brain development. Hence, we chose to investigate potential interaction between these two proteins and NFIs. To this end, we performed GST pull down assay and co-immunoprecipitation experiments and assayed for binding.





2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and commercial kits

All chemicals and commercial kits used in this research are listed in Appendix A.

2.1.2 Equipments

All equipments used in this study are listed in Appendix B.

2.1.3 Enzymes and Buffers

All enzymes and buffers used in this research are listed in Appendix C.

2.1.4 Media

2.1.4.1 Complete medium for HEK293T cell line

Complete medium consist of some ingredients, 440 ml DMEM, 50 ml Fetal Bovine Serum (FBS), 5 ml L-Glutamine (L-Gln) (100X), 5 ml Penicillin/Streptomycin (P/S) (100X), prepared by mixing and stored at 4°C.

2.1.4.2 Freezing medium for HEK293T cell line

Freezing medium consist of some ingredients, 4 ml DMEM, 1 ml DMSO and 5 ml of FBS, sterilized with 0.22 μ m filter and stored at -20°C.

2.1.4.3 Antibiotic free medium for transfection with PEI

Antibiotic free medium consist of some ingredients, 44.5 ml DMEM, 5 ml FBS, 0.5 ml L-Gln (100X), prepared by mixing and stored at 4°C.

2.1.5 Bacterial culture media

In this research, LB medium, LB agar medium and 2xYT medium were used to cultivate bacterial cells. Suitable antibiotic was added to each of the medium after sterilization to get selective culture condition. Antibiotics and their stock and final concentrations are listed in Table 2.1.

Table 2.1: Antibiotics used to obtain selective culture

	Stock concentration	Final concentration
Ampicillin	100 mg/ml	100 µg/ml

2.1.5.1 LB medium

10 gr of NaCl, 10 gr of tryptone, 5 gr of Yeast extract were dissolved in 900 ml dH₂O. Components were dissolved by stirring, and volume was completed to 1 L. Medium was sterilized by autoclaving at 120°C for 15 min.

2.1.5.2 LB-agar medium

10 g of NaCl, 10 gr of tryptone, 5 g of yeast extract and 15 g of agar were combined and dissolved in 900 ml dH₂O by stirring. Total volume was completed to 1 L. Medium was sterilized by autoclaving at 120°C for 15 min.

2.1.5.3 2xYT medium

10 g of yeast extract, 16 g of tryptone, 5 g of NaCl were dissolved in 900 ml dH₂O by stirring. pH was adjusted to 7 and volume was completed to 1 L. Medium was sterilized by autoclaving at 120°C for 15 min.

2.1.6 Bacterial strains

2.1.6.1 DH5α

DH5α is a chemically competent strain of *E. coli* and was used for replication of plasmids to further use in overexpression studies. This competent cell strain is also deficient in restriction and intracellular endonucleases.

2.1.6.2 BL21

BL21 is a chemically competent strain of *E. coli* which is deficient in Lon and OmpT proteases and this competent *E. coli* strain was used to express eukaryotic proteins at high level to further use in GST pull down experiments.

2.1.6.3 Stellar competent cell

HST08 strain of *E. coli* (Clontech, PT5055-2) is chemically competent and was used for transformation of our construct that are obtain by In-Fusion Cloning reaction. This strain provides high transformation efficiency.

2.1.7 Solutions

2.1.7.1 1M DTT

1,54 gr of DTT (Dithiothreitol, Mw:154,253 gr) was dissolved in total 10 ml dH₂O to obtain 1M DTT solution. Solution was aliquoted as 1ml and stored -20°C.

2.1.7.2 2M Glucose

7,2 g of glucose (Mw: 180,16) was dissolved in 20 ml dH₂O to obtain 2M glucose. This solution was sterilized by filtration through 0,2 µm filter. Solution was aliquoted as 1 ml and stored -20°C.

2.1.7.3 100 mM IPTG

25 mg of IPTG (Mw:238,30 gr) was dissolved in 1 ml dH₂O to get 100 mM IPTG. IPTG was sterilized by using filter and stored at -20°C.

2.1.7.4 5X SDS sample buffer

For expression control of proteins, 5X sample buffer was mixed with sample to loading SDS Polyacrylamide gel. Contents of 5X Sample Buffer are given in Table 2.2.

Table 2.2: Contents of the 5X Sample Buffer

Contents	Amount	Final Concentration
0,5 M Tris-Cl	600 µl	300 mM
100% Glycerol	600 µl	40% (v/v)
10% SDS	0.1 gr	0.01% (w/v)
BMP (10mg/ml)	0.1 gr	0.01% (w/v)

2.1.7.5 1.5X SDS sample buffer

For elute the proteins that bound to affinity resin, 1.5X SDS sample buffer was used. Contents of 1.5X Sample Buffer are given in Table 2.3.

Table 2.3: Contents of 1.5X Sample Buffer

Contents	Amount	Final concentration
100% Glycerol	150 µl	15%
0.5 M Tris HCl	187.5 µl	93.75 mM
10% SDS	300 µl	3% (v/w)
BMP(10 mg/ml)	75 µl	0.075 mg/ml
dH ₂ O	287.5 µl	

2.1.7.6 1.5 M Tris HCl (pH 8.8)

90.825 g of Tris base (MW: 121.14 g/mol) was dissolved in 400 ml dH₂O and pH was adjusted to 8.8 with HCl. Then bring up to volume 500 ml with dH₂O. Buffer was sterilized by autoclaving.

2.1.7.7 1 M Tris HCl (pH 7.5)

121.1 g of Tris base (MW: 121.14 g/mol) was dissolved in 800 ml dH₂O and pH was adjusted to 7.5 with HCl. Then bring up to volume 1L with dH₂O. Buffer was sterilized by autoclaving.

2.1.7.8 0.5 M Tris HCL (pH 6.8)

18.15 g of Tris base (MW: 121.14 g/mol) was dissolved in 200 ml dH₂O and ph was adjusted to 6.8 with HCl. Then bring up to volume 300 ml with dH₂O. Buffer was sterilized by autoclaving.

2.1.7.9 10% SDS

30 g SDS was dissolved in 300 ml dH₂O.

2.1.7.10 0.1% SDS

0.1% SDS solution was prepared by diluting of 10% SDS. 1ml of SDS (10%) was completed to 100 ml by adding dH₂O.

2.1.7.11 Ponceau S solution

Ponceau S solution was used for staining of nitrocellulose membrane to check transfer efficiency. Contents of solution are shown in Table 2.4.

Table 2.4: Contents of the Ponceau S solution

Contents	Amount	Fianl concentration
Ponceau S	0.5 g	0.5% (w/v)
Acetic acid	1 ml	1% (v/v)
dH ₂ O	Up to 100 ml	

2.1.7.12 10X Running buffer

30.2 g Tris base (MW: 121.14 g/mol) and 144 g Glycine (MW:75.06 g/mol) were dissolved in 800 ml dH₂O. Bring up to volume 1L with dH₂O.

2.1.7.13 1X Running buffer

1X Running buffer was prepared by diluting of 10X Running buffer. 90 ml 10X Running buffer and 10 ml 10% SDS were mixed and total volume was completed to 1L by addition of 900 ml dH₂O.

2.1.7.14 Blocking buffer

To prevent non-specific binding of antibodies to the membrane, blocking buffer was used. 1.5 g of skim milk powder was dissolved in 50 ml TBS-T.

2.1.7.15 10% Ammonium persulfate

1 g of Ammonium persulfate was dissolved in 10 ml dH₂O.

2.1.7.16 Coomassie blue staining and de-staining solutions

Coomassie blue staining solution was used to stain protein bands separated by SDS-PAGE. Stain was moved away by de-staining solution. Contents of solution are listed at Table 2.5.

Table 2.5: Contents of Commasie blue staining and de-staining solution.

Contents	Staining		De-staining	
	Amount	Final concentration	Amount	Final concentration
Acetic acid	10 ml	10% (v/v)	10 ml	10% (v/v)
Methanol	50 ml	50% (v/v)	50 ml	50% (v/v)
Coomassie Brilliant Blue (G-250)	0.1 g	0.1(w/v)	---	---
dH ₂ O	Up to 100 ml		Up to 100 ml	

2.1.7.17 1X Transfer buffer

100 ml 10X Running buffer which is described in section 2.1.7.12 and 200 ml methanol (final concentration 20%) were mixed and final volume was completed with 700 ml dH₂O for total 1L solution.

2.1.7.18 1X TBS and 1X TBS-T

Contents of the 1X TBS and 1X TBS-T solution are listed in Table 2.6.

Table 2.6: Contents of the 1X TBS and 1X TBS-T

Components	TBS		TBS-T	
	Amount	Final concentration	Amount	Final concentration
1M Tris pH 7.5	50 ml	50 mM	50 ml	50 mM
5M NaCl	30 ml	150 mM	30 ml	150 mM
Tween20	---	---	1 ml	0.1%
dH₂O	Up to 1L		Up to 1L	

2.1.7.19 5M NaCl

58,44 grams of NaCl (MW: 58,44) was dissolved in 160 ml dH₂O by stirring and volume was completed to 200 ml.

2.1.7.20 1X Sample buffer for whole cell extraction

Contents are listed in Table 2.7.

Table 2.7: Contents of 1X Sample buffer

Contents	Amount	Final concentration
0.5M Tris-HCl (pH: 6.8)	1.25 ml	62.5 mM
SDS (10% (w/v))	2 ml	2% (w/v)
Glycerol	1 ml	10% (w/v)
dH₂O	Up to 10 ml	

2.1.7.21 NP-40 Lysis buffer

NP40 Lysis Buffer was used for lysis of cells to be analyzed for protein-protein interaction. Before using NP-40 Lysis buffer protease inhibitor cocktail (25X) was added. Contents of the NP-40 lysis buffer are listed in Table 2.8.

Table 2.8: Contents of the NP-40 lysis buffer

Contents	Amount	Final concentration
1M Tris-HCl pH 7.5	10 ml	50 mM
NP40	2 ml	1% (v/v)
5M NaCl	6 ml	150 mM
0.5 M EDTA	2 ml	5 mM
dH₂O	Up to 200 ml	

2.1.7.22 50X and 1X TAE

For preparation of 50X TAE, 242 g of Tris base (MW: 121.14 g/mol) was dissolved in 750 ml dH₂O, then 57.1 ml of glacial acetic acid and 100 ml of 0,5 M EDTA (pH:8) were added and volume was completed to total 1L. 1X TAE was prepared by diluting of 50X TAE. For dilution, 20 ml of 50X TAE was combined with 980 ml dH₂O.

2.1.8 Components of the SDS polyacrilamid gel

Gel contents, used in this research are listed in Table 2.9.

Table 2.9: Separating and stacking gel contents.

Contents	Separating gel			Stacking gel
	10%	12%	15%	4%
Acrylamide solution	2.5 ml	3 ml	3.75 ml	0.5 ml
1.5M Tris-HCl pH: 8.8	2.5 ml	2,5 ml	2.5 ml	---
0.5M Tris-HCl pH: 6.8	---	---	---	1.25 ml
10% SDS	100 µl	100 µl	100 µl	50 µl
dH ₂ O	4.9 ml	4.5 ml	3.65 ml	3.2 ml
10 % APS	100 µl	100 µl	100 µl	50 µl
Temed	4 µl	4 µl	4 µl	5 µl

2.1.9 Antibodies

Antibodies are listed in Table 2.10. To protect antibodies against bacterial contamination, 0.02% sodium azide was added into working dilution.

Table 2.10: Antibodies, catalog numbers (Cat No) and working dilutions (WR).

Name	Cat No	WR
Polyclonal DYDDDDK Tag Antibody	CST 2368	1:500
HA-Tag monoclonal antibody Clone C29F4	CST 3724	1:1000
Anti-HA tag antibody polyclonal	Ab9110	1:1000
Anti-GST antibody polyclonal	Ab19256	1:1000
Anti-HA antibody monoclonal Clone 12CA5	Roche	1:500
Stabilized Peroxidase Conjugated Goat Anti-Rabbit (H+L)	Thermo Scientific, 32430	1:1000
Stabilized Peroxidase Conjugated Goat Anti-Mouse (H+L)	Thermo Scientific, 32460	1:1000
Veriblot for IP secondary antibody	Abcam, 131366	1:1000

2.2 Methods

2.2.1 PCR Primer Design for In-Fusion Cloning

For overexpressing target proteins which are ATXN3 and MAD2B, in-Fusion HD Cloning Kit (Clontech, 011614) was used to clone ATXN3 and MAD2B genes into pGEX-4T-3 bacterial expression vector and, pcDNA and pCMV mammalian expression vectors. These genes were cloned into the plasmids with suitable tags, FLAG, HA, Tomato and GST. In-Fusion PCR Primers were designed according to the instructions in In-Fusion® HD Cloning Kit User Manual. In-Fusion primers were designed to generate PCR products containing ends that were homolog to the ends of linearized vector. Cutting zones of enzymes on backbone illustrated at Figure 2.1.

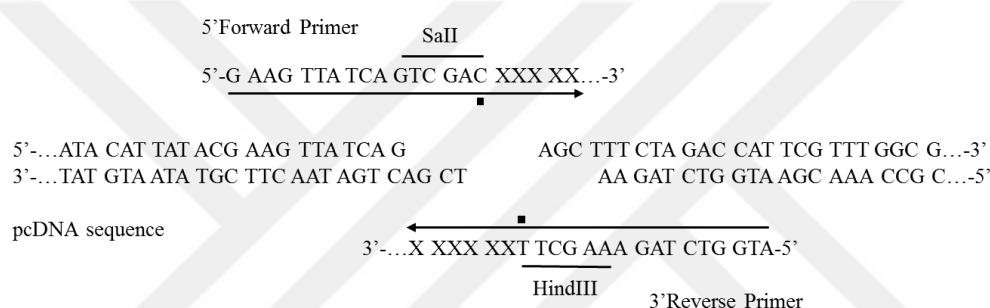


Figure 2.1: An example of double cutting zone on pcDNA. Forward and Reverse primers are include cutting zone sequence.

For successful insertion of a PCR fragment into the vector, PCR primer must share 15 base of homology with the ends of the linearized vector. The 5' end of the primer must contain 15 bases that are homologous to 15 bases at one end of the linear vector. The 3' end of the primer must contain sequence that is specific to the target gene. Homolog sequence is added to insert through the PCR primers. Part of sequences of these PCR primers were spesific for our target genes and vectors, named as identical to target sequence and not identical to target sequence respectively. Identical primer sequence contain our target gene spesific locus, however not identical part contain lineer vector cutting zones sequences. Primers which were used for amplification process listed at Table 2.11.

Table 2.11: List of in-fusion PCR primers

Primer name	Sequence	T _m (°C)
FLAG-ATXN3		55°C
FLAG_ATXN3_F (35 mer)	TGACGACAAGTGTACAATGGAGTCCATCTTCCACG	
FLAG_ATXN3_R (38 mer)	AAACGGGGCCCTCTAGATGGGTATTATTTTTTTCCTTCTG	
GST-ATXN3		55°C
GST_ATXN3_F (35 mer)	GGTTCCGCGTGGATCCATGGAGTCCATCTTCCACG	
GST_ATXN3_R (36 mer)	ATGCGGCCGCTCGATTATTTTTTTCCTTCTGTTTTCA	
HA-ATXN3		55°C
HA_ATXN3_F (37 mer)	CCAGATTACGCTCGAGAGATGGAGTCCATCTTCCACG	
HA_ATXN3_R (37 mer)	TAGACTCGAAGGATCCGGGTATTATTTTTTTCCTTCTG	
TOMATO-ATXN3		55°C
TOMATO_ATXN3_F (34 mer)	ATGGACGAGCTGTACATGGAGTCCATCTTCCACG	
TOMATO_ATXN3_R (38 mer)	ATTGGCGCCGCGGCCTTATTTTTTTCCTTCTGTTTTCA	
FLAG-MAD2B		58°C
FLAG_MAD2B_F (34 mer)	TGACGACAAGTGTACAATGACCACCCTCACGCGC	
FLAG_MAD2B_R (35 mer)	AAACGGGGCCCTCTAGATCAGCTGCCTTTATGAGCG	
TOMATO_MAD2B		58°C
TOMATO_MAD2B_F (33 mer)	ATGGACGAGCTGTACATGACCACCCTCACGCGC	
TOMATO_MAD2B_R (34mer)	ATTGGCGCCGCGGCCTCAGCTGCCTTTATGAGCG	

2.2.2 In-Fusion PCR Primer Dilution

We were ordered in-fusion PCR primers from Macrogen as lyophilised. These lyophilised primers were diluted with dH₂O reach up to 10 μ M was enough to use as PCR primers. Firstly, we were prepared 100 μ M primer as a stock after that 10 μ l stock primer was diluted at the rate of 1/10 (10 μ l 100 μ M primer + 90 μ l dH₂O). Consequently we were obtained 10 μ M primer dilution.

2.2.3 Preparation of a Linearized Vector by Restriction Digestion

To generate In-fusion experiment successfully, we must first generate a linearized vector. This linearized vector was generated using restriction enzymes with double digests. Increasing the enzyme digestion time and the digestion reaction volume will reduce the background. General protocol of preparation of a linearized vector by restriction enzyme digestion; 5 μ g of vector, 5 μ l buffer and 0,5 μ l enzyme(for each one) were enough for digestion in total 50 μ l reaction. We had to used suitable buffer for

enzymes that were linearized the vector. After that we were incubated our restriction digest as directed by the restriction enzyme supplier. Incubation from 3 hours to overnight at 37°C could increase linearization and reduce background. After digestion, we were purified the linearized vector using gel purification kit (NucleoSpin Gel and PCR Clean-Up Kit) to be explained in section 2.2.8. Components of the restriction digestion listed at Table 2.12.

Table 2.12: Components of Restriction Digestion.

Components	Volume/Reaction	Negative control Volume/Reaction
Total vector	Conc. of vector 5µg	Conc. of vector 5µg
Buffer	5.0µl	5.0µl
Enzyme	0.5µl	-
dH ₂ O	Up to 50µl	Up to 50µl

2.2.4 PCR Amplification of Target Sequence for In-Fusion Cloning

For the PCR amplification, we were used CloneAmp HiFi PCR Premix (sold in In-Fusion HD Plus Systems, and separately as Cat. No. 639298, ClonTech), which provides exceptionally accurate and efficient DNA amplification. The amount of time required for the amplification step has been standardized by Serial Cloner program. We were compared primers T_m values that were obtained from serial cloner and macrogene system. After comparison, we were decided to most suitable PCR condition and run PCR on the serial cloner program. Components of the PCR reaction are listed below.

Table 2.13: Components of PCR reaction.

Components	Volume/Reaction	Negative control Volume/Reaction
CloneAmp HIFI PCR Premix	12.5µl	12.5µl
F-Primer	5-7.5 pmol(0.2-0.3µM)	5-7.5 pmol(0.2-0.3µM)
R-Primer	5-7.5 pmol(0.2-0.3µM)	5-7.5 pmol(0.2-0.3µM)
Template	<100ng	-
dH ₂ O	Up to 25µl	Up to 25µl

The PCR reaction was first performed for two reaction volumes including one non-template negative control reaction. The size of the expected PCR product was

calculated by Serial Cloner biology software, and verified by agarose gel electrophoresis analysis. For the control of PCR reaction, 8 µl PCR product was mixed with 2 µl gel loading dye (New England BioLabs, #B7024S) and 10 µl mixture was loaded on agarose gel, together with 5 µl of MassRuler DNA Ladder Mix, ready-to-use (ThermoFisher Scientific). The gel was run at 90V for 50 minutes and observed under UV light to determine the size of PCR product. After the efficiency of In-Fusion PCR primers and reaction conditions were confirmed, PCR amplification was repeated again for four reaction volumes, in order to increase the amount of PCR product. Amplification products were purified from agarose gel using gel extraction kit to be explained in section 2.2.8. PCR temperature steps are listed at Table 2.14.

Table 2.14: PCR steps using CloneAmp HiFi Premix. For each primer sets, annealing temperatures were given on Table 2.11.

Initial Denaturaiton	98°C	10 sec	
Denaturation	98°C	10 sec	
Annealing	-----	30 sec	5 cycles
Extension	72°C	30 sec	
Denaturaiton	98°C	10 sec	30 cycles
Annealing&Extension	72°C	1 min	

2.2.5 In-fusion Cloning Reaction

In-Fusion cloning reaction was performed with In-Fusion®HD Cloning Kit (Clontech, Cat.#011614) according to the instructions in In-Fusion®HD Cloning Kit User Manual (Clontech). Base vector was selected and the insertion site was identified. The vector was linearized with restriction enzyme digestion. PCR primers were designed for our gene of interest with 15 bp extensions at the 5' ends that are complementary to the ends of the linearized vector. Purification of PCR products was performed with NucleoSpin Gel and PCR Clean-Up Kit. 100 ng linearized vector and 100 ng purified PCR fragment were mixed with 2 µl 5X In-Fusion®HD Enzyme Premix (Clontech) and total reaction volume was set to 10 µl by adding dH₂O. A negative control reaction lacking the PCR product was always performed accordingly. Reaction mixture was incubated at 50°C for 15 minutes, and then placed on ice. Products were used for the transformation procedures or stored at -20°C. Components and amount of each component used for cloning and negative control reaction are shown in Table 2.15.

Table 2.15: Components of In-Fusion cloning reaction.

Rxn Components	Cloning Reaction	Negative Control Reaction
Purified PCR Fragment	100 ng	-----
Linearized Vector	100 ng for 0.5- to 10 kb 50 ng for <0.5 kb	1 µl
5X In-Fusion HD Enzym Premix	2 µl	2 µl
Deionized Water	Up to 10 µl	Up to 10 µl

2.2.6 Construction of mammalian expression vector using in-fusion cloning

We used In-Fusion® HD Cloning Kit (Clontech, 011614), in order to construction of mammalian expression vector, We were designed new vector by cloning one or more DNA fragment into any vector. The main factor of In-Fusion cloning technology is Clontech's proprietary In-Fusion Enzyme. This enzyme fuses DNA fragments such as PCR-generated sequences and linearized vectors. This fusing process completed efficiently and precisely by recognizing a 15 bp overlap at their ends. 15 bp overlap ends can be engineered by designing primers for amplification of the desired sequences. PCR reaction is performed to amplify target sequence. Along with target sequence, additional sequences at both ends which homologous to the ends of the linearized plasmid was amplified by PCR. Just after purification and quantification of PCR product and linearized vector, in-Fusion reaction is set up. After then, a part of the reaction sample is transformed into suitable competent cells. The table below is a general outline of the protocol used for the In-Fusion HD Cloning Kits.

Table2.16: Outline Protocol to Generate New Construct

Step	Action
1	Basic vector selection and identification the insertion site. Linearize the base vector by restriction enzyme digestion.
2	Design PCR primers for our gene of interest with 15 bp extensions (5') that are complementary to the ends of the vector.
3	Amplify gene of interest and verify on an agarose gel
4	Purify PCR product
5	Set up your In-Fusion cloning reaction
6	Transform competent cells with 2.5 µl of the reaction mixture from Step 5.

General outline is used for generate new construct with In-Fusion HD Cloning Kits, illustrated in Figure 2.2.

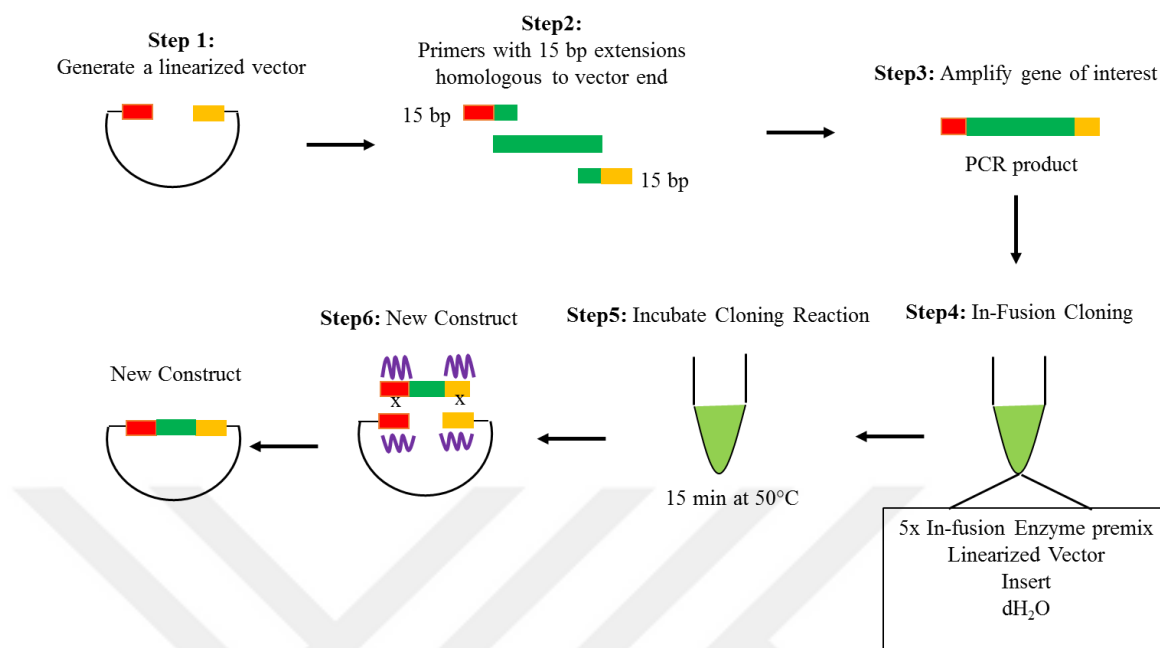


Figure 2.2: Schematic representation to construction of new expression vector.

2.2.6.1 Construction of pcDNA3.1-HA-ATXN3 expression vector

ATXN3 was cloned into HA-LMO4 vector which is previously designed in our lab (Saritaş, 2016) via In-Fusion cloning kit. HA-LMO4 vector was digested with BamHI and XhoI restriction enzymes and created linearized vector. ATXN3 was generated by amplification of MSCV-N-FLAG-HA-IRES-PURO (Plasmid #22538 Addgene, Sowa et al., 2009) vector was a gift from Wade Harper, using primers named as ATXN3_F and ATXN3_R listed in Table 2.11. Specific sequence of ATXN3 gene and homolog sequence to linearized vector ends that are both required for amplification of target sequence. Therefore, recombination of vector and amplifying product processes realized with these homolog ends. Besides that, additional sequences were rationally inserted within the primers into the vector. Kozak consensus sequence was affixed to forward primer in order to initiate translation of target protein in mammalian cells efficiently. HA tag sequence was added to N-terminus of target protein to create protein with a common affinity purification tag. Additionally, some cutting zone inserted into vector by primers to simplify further In-Fusion reactions. BamHI enzyme recognition sequence was placed between HA tag and ATXN3 sequences. In addition to this, XhoI enzyme recognition sequence was placed within the downstream sequence of ATXN3 via the reverse primer. Just before in-fusion cloning reaction,

linearized HA-LMO4 vector that was restricted with XhoI and BamHI enzymes and PCR product of ATXN3 were purified from agarose gel. After then, in-Fusion cloning reaction was set up. To initiate in-fusion reaction, 100 ng of linearized vector and 100 ng of purified ATXN3 PCR product were used. When in-fusion reaction completed, *E.coli* dh5 α competent cells transformed with 2,5 μ l of cloning reaction. Transformed cells spreaded on plate and a few colonies obtained from positive plate. These colonies were re-streaked and grown in 5ml LB medium containing Ampicillin at 37°C for overnight. Plasmid DNA isolation from selected colonies was done with miniprep kit. Quality of generated plasmid sequence was checked with restriction digestion. After then, newly created plasmid sequence verified by sequencing.

2.2.6.2 Construction of pcDNA3.1-FLAG-ATXN3 expression vector

ATXN3 was cloned into FLAG-PIN vector which is previously designed in our lab (Saritaş, 2016) and used as backbone and digested with BsrGI and XbaI enzymes. ATXN3 was designed with FLAG affinity tag on its N-terminus end. ATXN3 was amplified from MSCV-N-FLAG-HA-IRES-PURO vector using primers listed in Table 2.11 named as FLAG_ATXN3_F and FLAG_ATXN3_R. Before in-fusion cloning, amplification product and linearized vector were purified from agarose gel. To initiate in-fusion reaction, 100 ng of linearized vector and 100 ng of PCR product were used. *E.coli* dh5 α competent cells transformed with 2,5 μ l of cloning reaction. Transformed cells spreaded on LB agar containing Ampicillin and grown at 37°C for overnight. After then, selected colonies obtained from positive plate were re-streaked and grown overnight at 37°C. For miniprep experiment, selected colony grown in 5ml LB medium containing Ampicillin. After miniprep experiment, sequence of constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.6.3 Construction of pcDNA3.1-FLAG-MAD2B expression vector

MAD2B was cloned into pcDNA3.1-FLAG-PIN1 (Saritaş, 2016) with FLAG affinity tag on its N-terminus end. pcDNA3.1-FLAG-PIN1 vector used as backbone and linearized with BsrGI and XhoI enzymes. MAD2B gene was amplified from pGEX4T3-GST-LMO4 vector (Saritaş, 2016) using primers listed in Table 2.11 named as FLAG_MAD2B_F and FLAG_MAD2B_R. Before in-fusion reaction, amplification product and linearized vector were purified from agarose gel. To initiate in-fusion reaction, 100 ng of linearized vector and 100 ng of PCR product were used.

E.coli dh5 α competent cells transformed with 2,5 μ l of cloning reaction. Transformed cells spreaded on LB agar containing Ampicillin and grown at 37°C for overnight. After then, selected colonies obtained from positive plate were re-streaked and grown overnight at 37°C overnight. For miniprep experiment, selected colony grown in 5ml LB medium containing Ampicillin. After DNA isolated via miniprep experiment, sequence of constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.6.4 Construction of tdTomato-ATXN3 expression vector

For overexpress ATXN3 protein with an fluorescent tag on its N-terminus end, ATXN3 was cloned into pCMV_TdTomato vector which was a gift from Gerhart Ryffel (Addgene plasmid #30530, Waldner et al, 2009). To linearize pCMV_TdTomato vector, BsrGI and NotI restriction enzymes were used. ATXN3 was amplified from pcDNA3.1-HA-ATXN3 vector using primers which are listed in Table 2.11 named as TdTomato_ATXN3_F and TdTomato_ATXN3_R. To initiate infusion reaction, 100 ng of vector and 100 ng of PCR product were used. Constructed plasmid DNA was isolated via miniprep kit and checked with restriction digestion. After then, plasmid DNA was verified by sequencing.

2.2.6.5 Construction of tdTomato-MAD2B expression vector

For overexpress MAD2B protein with an fluorescent tag on its N-terminus end, MAD2B was cloned into pCMV_TdTomato empty vector. To linearize pCMV_TdTomato vector, BsrGI and NotI restriction enzymes were used. MAD2B was amplified from pcDNA3.1-FLAG-MAD2B vector using primers listed in Table 2.11 named as TdTomato_MAD2B_F and TdTomato_MAD2B_R. To initiate infusion reaction, 100 ng of vector and 100 ng of PCR product were used. Constructed plasmid DNA was isolated via miniprep kit and checked with restriction digestion. After then, newly generated plasmid was verified by sequencing.

2.2.7 Construction of pGEX4T3-GST-ATXN3 expression vector

For overexpress ATXN3 in *E.coli* BL21 cells for GST Pull down experiments, cDNA encoding ATXN3 cloned into pGEX4T3 bacterial expression vector. To linearize pGEX4T3 vector, BamHI and XhoI were used. ATXN3 was amplified from pcDNA3.1-HA-ATXN3 vector which was constructed from MSCV-N-FLAG-HA-IRES-PURO (Plasmid #22538 Addgene) using primers listed in Table 2.11 and named

as pGEX4T3_ATXN3_F and pGEX4T3_ATXN3_R. Before in-fusion reaction, digested vector and PCR product were purified from agarose gel. To initiate in-fusion cloning, 100 ng of vector and 100 ng of amplified product were used. *E.coli* BL21 competent cells transformed with 2,5 µl of cloning reaction. Colonies obtained from positive plate were re-streaked on LB agar containing Ampicillin, grown at 37°C for overnight. Constructed plasmid DNA was isolated via miniprep kit. Quality of generated plasmid sequence was checked with restriction digestion. After then, newly created plasmid sequence verified by sequencing.

2.2.8 Analytic Gel Electrophoresis to detection of PCR products or vectors

To check the accuracy of PCR product and vectors, analytic gel electrophoresis was performed. For PCR product: 1 µl of PCR product mixed with 1 µl loading dye (New England BioLabs, #B7024S) and loaded into 0.8% Agarose gel well together with 5µl of MassRuler DNA Ladder (Thermo Scientific, #SM0403). For vector products same process was performed. 1 µl of linearized vector was mixed with 1 µl loading dye and loaded into agarose gel wells. The gel, loaded with PCR products or vector with marker was run at 90V for 50 minutes. To observe the position of the bands, gel exposed UV light. In-Fusion cloning reaction was set according to these agarose gel results.

2.2.9 Agarose Gel Extraction of PCR products and linearized vectors

NucleoSpin Gel and PCR Clean Up Kit (Macherey-Nagel, REF. 740609.50) was used for extraction of DNA fragments from agarose gel just after accuracy process. Total 100 µl PCR product was mixed with 20 µl loading dye (New England BioLabs, #B7024S) and loaded into the agarose gel well together with MassRuler DNA Ladder (Thermo Scientific, #SM0403). In addition to PCR products, linearized vector also extracted from agarose gel. 50 µl linearized vector was mixed 10 µl loading dye (New England BioLabs, #B7024S) and this mix was loaded into the agarose gel well together with MassRuler DNA Ladder (Thermo Scientific, #SM0403). When samples loaded in to the well together with marker, gel was run at 90V for 50 minutes. The position of DNA sample and marker were determined under UV light and the location of band to be removed from agarose gel was detected. The band was precisely cut out from gel, avoiding too long exposure to UV light. The gel band with desired DNA sample was weighted in a microcentrifuge tube. Just before weighing, micro centrifuge tube was weighted alone as empty and tube weight was determined. After then, the amount

of excised gel was determined as 700 mg. In the case of gel intensity <2%, for each 100 mg of agarose gel, 200 µl buffer NTI was added on gel. Just after, the sample was incubated at 50°C on heating block for 5- 10 minutes. During heating process, the gel was dissolved by vortexing every 2-3 minutes. When gel sample dissolved completely, dissolved gel sample was loaded on NucleoSpin Gel and PCR Clean Up column. Loaded column centrifugated at 11000 x g for 60 seconds, in order to discard dissolved gel. After centrifugation, flow through was removed and column washed with 700 µl of wash buffer NT3. To remove wash buffer from column, sample was centrifuged at 11000 x g for 60 seconds and the flow through was discarded. This wash step was repeated, in order to discard the remaining of buffer NT3 completely by centrifugation at 11000 x g for 1 minute. Before elution step, ethanol was removed from sample by incubation at 70°C on heating block for 2-3 minutes. When remaining ethanol removed from sample, column was placed in a clean collecting tube. 15-30 µl of elution buffer NE (5 mM Tris/HCl, pH 8.5) was pre-heated (up to 70°C) and added on column. The sample was incubated at room temperature for 1 minute. Just after incubation process, column was centrifuged at 11000 x g for 1 minute in order to elute DNA. The concentration and purity of gel PCR product and linearized vector were determined with absorbance (A260) measurements.

2.2.10 Transformation

2.2.10.1 Stellar Competent Cells

Stellar Competent Cells procured by In-Fusion®HD Plus Kit. These competent cells were used for the transformation of In-Fusion cloning products. Stellar Competent Cells were stored at -80°C as 100 µl amounts in the micro centrifuge tubes. Just before transformation, Stellar Competent Cells taken out from -80°C and were dissolved on ice by pipetting gently. Dissolved Stellar Competent Cells were divided into 2 micro centrifuge tubes as 50 µl x 2. Cells in the first tube were going to be used as positive control and these cells transformed with In-Fusion reaction product, and the cells in the second tube were used as negative control of In-Fusion reaction. For each tubes, 2.5µl In-Fusion reaction product added onto the 50 µl Stellar Competent Cells (with negative control In-Fusion reaction for control transformation) and these transformation reaction mixtures incubated on ice for 30 minutes. After incubation on ice, cells were heat shocked at 45°C (on heating block or in water bath) for 60 seconds (for Eppendorf tubes) to stopped reaction. When cells heat shock process completed ,

tubes were incubated on ice for 5 minutes. Transformation reaction mixture completed up to 500 µl by adding 450 µl SOC Medium (Clontech, ST0215), which was dissolved in room temperature. After then, tubes were incubated at 37°C for 90 minutes with 160-225 rpm agitation.

To growth transformed cells as colony, LB agar plates containing appropriate antibiotic (Ampicillin) were used. Before using, LB agar plates was pre-heated to RT. 100 µl of transformed cell culture was directly spread on LB plate containing appropriate antibiotic. When spreading process completed, this plate was named as 1:5 and made into upside down. Remaining 400 µl of cell culture was centrifuged at 6000 rpm for 5 minutes. After cells collapsed on bottom, 300 µl of supernatant was discarded and the pellet was re-suspended in 100 µl supernatant. This concentrated mixture was spread on a separate LB-antibiotic plate, which was named as 4:5 and made into upside down. Same plating steps were followed for negative control transformation reaction which was performed with negative control In-Fusion reaction. 4 plates (1:5 Sample, 4:5 Sample, 1:5 Negative Control, 4:5 Negative Control) were placed upside down and incubated at 37°C for an overnight (16 hours). When incubation completed, 6 colonies selected from the sample and negative control plates. These colonies were re-streaked on 2 LB-antibiotic plates and incubated overnight at 37°C in upside down position. Caps of the petri plates were sealed with Parafilm and the plates were stored at -20°C in plastic bags, for later use.

2.2.10.2 *E. coli* DH5α cells

For transformation of mammalian expression vectors, *E.coli* DH5α competent cells were used. Before using, LB medium and agar plates were preheated to RT. *E. coli* DH5α competent cells were used instead of Stellar Competent Cells. Thus, the amount of competent cells to be transformed was doubled. Therefore, 100 µl DH5α competent cells were transformed with 2.5 µl In-Fusion reaction product instead of 50 µl cells. *E.coli* DH5α competent cells were melted on ice just before use and 100 µl of cells were aliquoted directly into pre-chilled micro centrifuge tubes. 20 ng of plasmid DNA was added onto the cells and tube mixed gently. Just after DNA adding, tubes incubated on ice for 30 min. To ending reaction, heat shock was performed at 42°C for 1.5 min. After then, reaction was incubated on ice for 2 min. Finally, total reaction volume completed to 500 µl by adding 450 µl of pre-heated LB medium. Cells were incubated at 37°C, for 90 min on a shaker. After incubation process, cells plated on

agar plate containing appropriate antibiotic. Plates were placed upside down and incubated at 37°C for overnight.

2.2.10.3 *E. coli* BL21 cells

For the overexpression of GST tagged protein, *E. coli* BL21 cells were used. BL21 competent cells were thawed on ice just before use. 50 µl of thawed cells were aliquoted directly into pre-chilled micro centrifuge tube. 100 ng of plasmid DNA were added onto the cells, mixed gently and incubated on ice for 30 min. To stop reaction, heat shock was performed at 42°C for 1.5 min. After heat inactivation, tube was incubated on ice for 2 min. Subsequently, 450 µl of preheated LB medium was added onto the cells to complete the volume up to 500 µl. 500 µl cell suspension was incubated at 37°C, for 90 min on a shaker. When cells saturates, *E. coli* BL21 cells were plated on LB agar plate containing 20 mM glucose and appropriate antibiotic.

2.2.11 Miniprep plasmid DNA isolation

NucleoSpin® Plasmid Purification Kit (Macharey Nagel, REF:740588.50) was used for miniprep plasmid DNA isolation. For isolation of high-copy plasmid DNA from *E.coli*, NucleoSpin® Plasmid protocol was followed. Prior to plasmid isolation, Single DH5α colony was selected from there-streak plates of In-Fusion plasmid transformations. After then, selected colony inoculated into 5 ml LB broth media and was grown at 37°C with agitation for 8 hours,. When incubation time completed, 5 ml of *E. coli* culture was saturated. Saturated *E. coli* culture centrifuged at 11000 x g for 30 seconds to collapse cells on bottom and the supernatant was discarded. Collapsed *E. coli* cells harvested with 250 µl of Buffer A1 and the pellet was resuspended completely by vortexing and pipetting. 250 µl Buffer A2 was added and the tube was inverted 5-6 times to mix the components. Completely suspend *E. coli* cells mixture was incubated at RT for 5 minutes, until the cell lysate appeared clear. After re-suspension step, Cells were lysed with 300 µl Buffer A3. 300 µl Buffer A3 was added and the tube was inverted 6-8 times to mix the components. Cell lysis was completed by Buffer A1, A2 and A3, and the lysed sample was centrifuged at 11000 x g for 5 minutes, in order to clarify the lysate. After centrifugation cell clumps feel down on bottom and supernatant was load on NucleoSpin® Plasmid Column, which was placed in a 2 ml Collection Tube. Lysate was centrifuged through the column at 11000 x g for 1 minute and flow-through was removed. This loading and centrifugation steps were

repeated until all lysate was loaded on column. To remove all residual, an additional washing step was performed by adding 500 μ l Buffer AW, which was preheated to 50°C. Before proceeding with buffer A4, Buffer AW removed via centrifuging at 11000 x g for 1 minute. After silica membrane was initially washed with Buffer AW, 600 μ l Buffer A4 (supplemented with ethanol) was added on column and washing steps were over. The sample was centrifuged at 11000 x g for 1 minute to remove Buffer A4. After washing steps were over, the silica membrane was dried by centrifuging at 11000 x g for 2 minutes to remove residual washing solution. When column dried, NucleoSpin® Column was placed in a fresh 1.5 ml microcentrifuge tube and 50 μ l Buffer AE was added for elution step. Column was incubated at RT for 1 minute and then centrifuged at 11000 x g for 1 minute. Eluted miniprep DNA samples concentration and purity were determined by NanoDrop (Thermo Fisher Scientific) measurements, and restriction digestion analysis. These analyses were performed in order to check the miniprep samples for the presence of insert, before sequencing.

2.2.12 Restriction digestion and agarose gel analysis

Miniprep samples controlled with restriction digestion to check accuracy. Restriction digestion analyses performed with suitable enzymes that were used with NEBuffer. Restriction digestion reaction of 500 ng DNA sample was performed in a 20 μ l of total reaction mixture with 0.5 μ l suitable enzyme and 2 μ l 10X Buffer. Total reaction volume was completed with dH₂O. Single or double restriction cut reactions were performed at 37°C for 1 hour incubation. When restriction digestion completed, reaction ended with heat inactivation at 65°C for 20 minutes incubation. Contents of the restriction digestion process are listed at Table 2.17. The efficiency of restriction digestion was analyzed by agarose gel electrophoresis. 10 μ l of restriction digested miniprep plasmids samples were mixed with 2 μ l loading dye (New England BioLabs, #B7024S) and load on Agarose gel wells in the presence of MassRuler DNA Ladder (Thermo Scientific, #SM0403).

Table 2.17: Components of restriction digestion

Components	Volumes
DNA	500 ng
Buffer	2 µl
Enzyme	0,5 µl (double or one cut)
dH ₂ O	Up to 20 µl

2.2.13 Sequencing analysis

To check accuracy of constructed plasmids isolated by miniprep, restriction digestion analysis firstly used for the presence of insert. After then selected plasmids were sent to Macrogen for sequencing analysis. Generally, universal primers were selected for forward sequencing target sequence fusion plasmids. After the sequencing results obtained and checked, one of the miniprep plasmids, which was observed to be correct, sometimes constructed plasmid was sequenced with reverse universal primer if necessary. All sequencing analysis listed in Appendix D.

2.2.14 Bacterial growth for plasmid isolation

Single colony was selected from streaked plate and inoculated into the 5 ml of LB medium containing selective antibiotic. Starter culture incubated at 37°C for 16 hours on shaker at 200-225 rpm. After incubation, starter culture was used for miniprep preparation or diluted as 1:1000 to 1:10,000 ratio and inoculated to 250 ml LB medium containing appropriate antibiotic to precede maxiprep preparation.

2.2.15 Maxiprep plasmid DNA isolation

GeneJET Plasmid Maxiprep Kit (Thermo Scientific, K0492), NucleoBond®Xtra Midi/Maxi Kit (Macherey-Nagel, 740414.50) and Zippy Plasmid Maxiprep Kit (Zymo Research, Cat#D4027) were used for isolation of plasmid DNA.

2.2.15.1 Maxiprep using GeneJET plasmid maxiprep kit

E. coli cell culture was grown at 37°C for overnight and cells gathered by centrifugation at 5000xg for 10 min at +4°C. After centrifugation, supernatant was removed and pellet was re-suspended in 6 ml of resuspension solution including RNase A. Pellet solved in resuspension solution by vortexing until no cell clumps remain. After resuspension, 6 ml of Lysis solution was added onto resuspended pellet and mixed gently by

inverting the tube 4-6 times until the solution becomes viscous. Solution was incubated at room temperature for 3 min and not exceeded time limit. Immediately after, 6 ml of Neutralization Solution was added to stop reaction and mixed gently by inverting the tube 5-8 times. When reaction activity stopped by neutralization solution, 800 µl of Endotoxin binding reagent was added into mixture and mixed by inverting the tube 5-8 times. After mixing, tube was incubated at room temperature for 5 min and incubation timeout not exceeded. 6 ml of 96% ethanol was added into the tube and mixed by inverting the tube 5-6 times. Then, mixture centrifuged at 3200xg for 60 min. When cells clumps collapsed at the bottom of the tube, supernatant was transferred carefully to a fresh 50 ml tube by decanting or pipetting. 6 ml of 96% ethanol was added onto supernatant and mixed by inverting the tube 5-6 times. Approximately 20 ml sample solution was transferred to the column and centrifuged at 2000xg for 3 min. After centrifugation, flow-through was discarded. Remaining sample loaded to the column and centrifugation were repeated to discard flow-through. Column filter washed with 8 ml of wash solution I and centrifuged for 2 min at 3000xg. Flow-through was discarded from tube. Washing steps of column with solution repeated. 8 ml of wash solution II (including ethanol) was added to the column and centrifuged at 3000xg for 2 min and flow-through removed. Washing and centrifugation steps were repeated. After repeated washing steps, empty tube was centrifuged at 3000xg for 5 min to remove residual wash solution. Washed column was transferred to a fresh 50 ml tube (provided by kit). 1 ml of elution buffer was added to the center of the column. After then, column incubated at room temperature for 2 min, and centrifuged at 3000xg for 5 min. Quality and quantity control of plasmid DNA was checked by UV spectroscopy and restriction digestion. Eluted plasmid DNA was stored at -20°C.

2.2.15.2 Maxiprep using NucleoBond®Xtra midi/maxi kit

MN NucleoBond Xtra Maxi Plus Kit was used for maxiprep preparation. *E.coli* cell culture was grown at 37°C for overnight. After 16 hours incubation, cells harvested by centrifugation at 3000xg for 20 min at +4°C. When cells fell down, supernatant was discarded. Pellet collapse, bottom of the tube was resuspended in 12 ml Resuspension Buffer by vortexing until no cell clumps remain. After cells resuspend, 12 ml Lysis Buffer solution was added into the tube and mixed by inverting the tube for 5 times. Mixture was incubated at room temperature for 5 min. During incubation time, column filter equilibrated. To equilibrate column filter, the equilibration buffer applied onto the

rim of the column to wet completely. To stop reaction, 12 ml Neutralization buffer was added to suspension and mixed by inverting the tube 10 times until sample become colorless. After neutralization process, sample was applied to the equilibrated column and waited for getting through of lysate from column by gravity flow. After all sample clarified, column filter was washed with 15 ml equilibration buffer and filter was removed. The column was wash with 25 ml Wash Buffer during washing step. After column washed, plasmid DNA was eluted from column by adding 15 ml Elution Buffer and collected in a fresh 50 ml tube. To precipitate DNA, 10.5 ml isopropanol was added into the tube, and tube mixed thoroughly. Later on tube incubated for 2 min at room temperature. Syringe was attached to Nucleobond® Finalizer and filled with mixture. Sample passed through the Finalizer drop by drop manner, and flow-through was removed. Finalizer was washed with 5 ml 70% ethanol.,After then, Finalizer dried by pressing air as strongly as possible for at least 6 times. When drying process completed, Finalizer was placed on a fresh 1.5 ml micro centrifuge tube. Plasmid DNA was eluted with 500 µl Tris or dH₂O. First eluate was transferred back into the syringe and eluted into the same tube a second time to enhance yield. Quality and quantity of plasmid DNA was checked by UV spectroscopy and restriction digestion. Eluted plasmid was stored at -20°C.

2.2.15.3 Maxiprep using Zippy Plasmid Maxiprep Kit

Prior to maxiprep isolation procedure, *E. coli* DH5α cells were transformed with selected miniprep sample and a single colony was inoculated in 5 ml LB-Ampicillin Broth media for 8 hours with agitation. 5 ml DH5α culture was transferred to 100 ml fresh LB-Ampicillin Broth media and large culture was grown at 37°C for 16 hours with agitation. Zippy Plasmid Maxiprep Kit (Zymo Research, Cat.#D4027) was used for maxiprep plasmid DNA isolation. The isolation procedure was performed according to the protocol. For isolation of plasmid DNA, vacuum used. Fresh bacterial culture was centrifuged at 3400 x g for 10 minutes. When cells clumps collapsed, the supernatant was removed. To suspend pellet, 15 ml of P1 Buffer (Red) was added to the bacterial cell pellet. After then, the pellet was re-suspended completely by vortexing or pipetting. When cell pellet completely suspend, 15 ml of P2 Buffer (Green) was added on re-suspended culture and suspension mixed by inverting the tube 4-6 times. Cells were incubated with P2 Buffer for 1 minute at RT to lyse cell completely. To terminate lysis process, 20 ml of P3 Buffer was added on culture and

mixed gently. Lysed culture was incubated with P3 Buffer on ice for 5 minutes. When neutralization was completed, and a yellowish precipitate was observed, the sample changed its color to yellow. After neutralization process completed, Zymo-Maxi Filter/Zymo Spin VI column assembly was placed onto vacuum manifold. Lysed culture mixture was added into the blue Zymo-Maxi Filter column and the liquid was vacuumed through the columns. When the entire liquid vacuumed, Blue Zymo-Maxi Filter was discarded from the top of Zymo-Spin VI column. To wash spin column, 10 ml of Endo-Wash Buffer was added to Zymo-Spin VI column and vacuumed through the spin column. After first washing step completed and the Endo-Wash Buffer had completely passed through the Zymo-Spin VI column, 10 ml of Zyppy Wash Buffer was added on column and vacuum was turned on. The entire wash buffer vacuumed through the spin column. To remove all residual Zyppy Wash Buffer, vacuum was left on for an additional 5 minutes. When spin column completely dried, Zymo-Spin Column VI was transferred into a clean 50 ml conical tube and 2-3 ml of Elution Buffer (Thermo Scientific) was added to enter of the column for elution. Because of the EDTA content, Zyppy Elution Buffer was not used for elution step. Column was incubated with Elution Buffer at room temperature for one minute, then centrifuged at 3400 x g for 1 minute to elute the plasmid DNA. The concentration of maxiprep sample was determined by NanoDrop measurements. Eluted plasmid DNA was stored at -20°C.

2.2.16 Expression analysis of protein

2.2.16.1 Protein extraction from BL21 cell

BL21 *E.coli* cells were transformed with intended maxiprep samples. After transformation, *E.coli* cells plated on LB agar plates with suitable antibiotic. Later on, *E.coli* cells were grown at 37°C for 16 hours. After incubation, isolated colonies were selected from each transformation to induce cells. One colony picked up for IPTG induction and marked as IPTG+ and one colony picked up for the control and labelled as IPTG-. Selected colonies were inoculated in 3 ml of 2X YTA Medium (3 ml 2X YT Medium + 3 µl 1000x Ampicillin + 30 µl 100x Glucose). Inoculated cultures were incubated at 37°C for 3-5 hours on shaker and waited for the OD600 were determined. When OD600 value was reached up to 0.6-0.8, 2 µl of 1000x IPTG was added into culture and were incubated at 37°C for an additional one hour on shaker. Besides IPTG+ cultures, IPTG- control cultures were also incubated for additional one hour.

At the end of the incubation, IPTG + and IPTG – cultures were taken from 37°C and centrifuged at 6000 x g for 4 minutes. After clumps fell down, supernatant was removed and the pellet was dissolved in 150 µl Sample Buffer (Total 5 ml: 500 µl Glycerol, 1 ml 10% SDS, 62.5 µl 0.5M Tris, 2.9 ml dH₂O). To determinate expressed proteins, SDS PAGE and Western Blot analysis were performed.

2.2.16.2 Preperation of whole cell extract

48 hours after transfection of HEK293T cells, cells were ready for extraction. Whole-cell extracts were prepared using 1X Sample Buffer. Sample Buffer was warmed up to 95°C before use in extraction. 48 hours later transfection, transfected cells were washed twice with 1 ml PBS, and PBS is removed via aspiration carefully. Just after, transfected cells scraped with 400 µl 1X Sample Buffer. After scraping, lysates were boiled at 95°C for 10 min, and boiled lysates passed through a syringe for 5 times. Immediately after, lysates cleared by centrifugation at 12000xg for 10 min. When clump fell down, supernatants were collected and aliquoted in micro centrifuge tubes. Concentrations of each sample were determined by using BCA protein assay kit (Pierce), and then samples subjected to Western Blotting.

2.2.16.3 Membran extraction

48 hours after transfection of HEK293T cells, medium was removed and plate was washed two times with 1 ml PBS. Then, plate was incubated on ice and 1 ml precooled NP40 Lysis Buffer contained EDTA and protease inhibitor was added. After placed on ice for 30 min, plate was incised and lysate was gathered in a 1.5 ml precolled micro centrifuge tube. Lysate was centrifuged for 10 min at 10000xg at +4°C. Supernatant was aliquoted into fresh pre-chilled 1.5 ml micro centrifuge tubes and stored at -80°C.

2.2.17 Measurement of protein concentration

Protein concentrations were evaluated by using the bicinchoninic acid (BCA) protein assay kit (Pierce). This quantification procedure depends on the conversion of Cu⁺² ions to Cu⁺ ions in the case of the presence of four amino acid residues (cysteine, tyrosine, tryptophan and cystine) in peptide molecules. This reaction occurs in a temperature dependent manner. NEB-C buffer was used as a solvent for measurements of nuclear extract samples. To measurement of whole cell extract, 1X Sample buffer was added onto the samples. BCA protein standards were prepared as defined in Table 2.18

Table 2.18: Components of the BCA standards

Vial	Volume of diluent (μl)	Volume and source of BSA (μl)	Final BSA concentration
A	0	300 of Stock	2000 μ g/ μ l
B	125	375 of Stock	1500 μ g/ μ l
C	325	325 of Stock	1000 μ g/ μ l
D	175	175 of vial B dilution	750 μ g/ μ l
E	325	325 of vial C dilution	500 μ g/ μ l
F	325	325 of vial E dilution	250 μ g/ μ l
G	325	325 of vial F dilution	125 μ g/ μ l
H	400	100 of vial G dilution	25 μ g/ μ l
I	400	0	0 μ g/ μ l=Blank

Working reagent (WR) was prepared by diluting BCA reagent B in BCA reagent A at 1:50 ratio. In a 96-well microplate, first 200 μ l working solution was pipetted into each microplate well and then 25 μ l of BCA standards or unknown samples were added onto the working reagent; samples were shaken for 30 sec. Then, microplate was incubated at 37°C for 30 min. After incubation at 37°C, absorbance at 562 nm was measured on plate reader spectrophotometer (Shimadzu, UV-1601).

2.2.18 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE is a widely used molecular technique to separate and identify proteins according to electrophoretic mobility. SDS is an anionic detergent and used to break the tertiary structures of proteins. Besides that SDS decreases agents such as dithiothreitol (DTT) was used to removed all di-sulfide bonds. Before Western blot analysis, lysates were first resolved by SDS-PAGE. 1X Sample Buffer and 20X DTT added onto the proper amount of protein sample, then boiled at 95°C for 5 min. After boiling, samples were loaded into gel wells along with a protein standard and started running at constant 90 V, then carried at constant 110 V.

2.2.19 Western blotting

Western Blot analysis is performed to detect specific proteins in a sample. For Western blotting, denatured proteins mix was first analysed in the separating gel according to their size. After then, proteins were transferred onto nitrocellulose membranes using wet transfer system (Biorad). Just before transfer, nitrocellulose membrane and gel were equalized with 1X transfer buffer for 5 min. When the gel and membrane were equalized, these were sandwiched between sponges and filter papers (Whatman)

according to manufacturer's instructions. Air bubbles were removed during setting up transfer system. Sandwich system was inserted into the tank and run overnight at +4°C, 16V in 1X Transfer Buffer, previously equalized at +4°C. After blotting process completed, membrane bound proteins were made visible by Ponceau-S staining. After membrane dyed with Ponceau-S staining, lanes and positions of protein molecular weight markers were signed on the membrane. To prevent non-specific binding, the membrane was blocked with 3% skim milk powder in TBS-T for 1 h at RT. Right after, the membrane was washed twice with TBS-T for 5 min at RT and incubated with primary antibody that diluted in 10% FBS in TBST overnight at 4°C on a rotator. Following day, membrane was washed 3 times with TBS-T for 5 min. Then, membrane was incubated with proper secondary antibody prepared in 3% milk-powder in TBST for 1 hour at RT on shaker. After incubation with 3% milk-powder membrane washed 2 times with TBS-T, and once with TBS for 5 min. Protein bands were visualized using chemiluminescent substrate by ChemiDoc™ MP System (Biorad).

2.2.20 Immunoprecipitation

For immunoprecipitate our tagged proteins, we used α -FLAG® M2 affinity gel (Sigma A2220). 50% slurry 20 μ l suspension of the antibody conjugated agarose resin was added into the 1.5 ml micro centrifuge tube for each sample. Resin was washed with 1 ml NP40 Lysis Buffer by inverting the tube in each tube and centrifuged at 8000xg for 30 sec. When we were observed that resin was gravitated completely, supernatant was removed. Previously washed pellet washed with 2X1 ml NP40 Lysis Buffer and centrifuged at 8000xg for 30 sec. Appropriate amount (0.5-1 mg) of HEK293T cell lysate was transferred to settled resin, and incubated at +4°C for overnight on an orbital shaker. The following day, sample was centrifuged at 2000xg at +4°C for 2 min and supernatant was transported to a fresh micro centrifuge tube, and stored at -20°C. All washing steps were performed with 1 ml NP40 Lysis Buffer by turning inside out the tube and sample centrifuged at 2000xg for 2 min at +4°C. This process was repeated for three times. Supernatant was totally and intently removed after the last centrifugation. After that 50 μ l 1.5X Sample Buffer was implemented into each micro centrifuge tube. Immunoprecipitated proteins were denatured at 95°C by boiling for 5-10 min. Then, boiled proteins centrifuged at maximum g for 1 min at RT. After centrifugation supernatant was removed and added into a fresh micro centrifuge tube.

DTT is added into the sample if needed. Supernatant transferred into fresh micro centrifuge tube and stored at -20°C or resolved by SDS-PAGE.

2.2.21 GST pull-down

To pull down GST or GST tagged proteins, we used Glutathione-Agarose (Sigma, G4510) or Glutathione Sepharose 4B (GE 170756-01). At first, resin was washed three times with BugBuster. Then, 20% slurry Glutathione resin was prepared in Bugbuster solution and stored at +4°C. For each pull down experiment, 50 µl glutathione resin (20% bed volume) was put into a 1.5 ml microcentrifuge tube. Proper amount (10-20 µg) of BL21 cell lysates including GST or GST tagged proteins were added onto the glutathione resin and incubated for 50 min at RT on an orbital shaker. After then, HEK293T cell lysate were added onto glutathione resin, and incubated at +4°C for 2 hrs on an orbital shaker. Incubated resin and lysate mixture was centrifuged at 500xg at +4°C for 5 min. Flow through was transposed into a new micro centrifuge tube and store at -20°C. When flow through removed, pellet was washed three times with PBS containing 1% TritonX (v/v). After the last washing step with PBS, 50 µl 1.5X Sample Buffer was supplemented into each micro centrifuge tube, and pellet was boiled at 95°C for 5-10 min with 1.5X Sample Buffer. After all, micro centrifuge tube was centrifuged at max g for 1 min and eluate was transferred into a new micro centrifuge tube and examined with western blotting.

2.2.22 Cell culture methods

2.2.22.1 Thawing of HEK293T cells line

Cells were removed from liquid nitrogen storage and immediately transferred to a 37°C water bath and dissolved quickly with agitation. Then, thawed cells were transported into 15-ml sterile centrifuge tube. 4 ml pre-warmed medium (Dulbecco's Modified Eagle Medium (DMEM), %10 FBS, 2 mM Glutamine, 100 u/ml Penisilin/100 mg/ml Streptomisin) carefully drop-wise. An additional 5 ml pre-heated complete medium was added into the same tube. Cells were centrifuged for 5 min at 300xg and supernatant was removed. Pellet was dispersed in remaining medium by tapping the tube. 10 ml fresh pre-warmed medium was added onto the cells and cells were plated into 100 mm plate dish. Plate was placed into a 37°C incubator with 5% CO₂. Next day, the medium was replaced with the fresh pre-warmed complete

medium. When the culture was seen 90% confluent under microscope, cells were passaged.

2.2.22.2 Subculturing of HEK293T cell line

For that purpose, Cell-dissociation reagent (0.25% Trypsin-EDTA solution) and the complete medium were pre-warmed to 37°C. Firstly, the complete medium onto the cells was removed from the cells by aspiration. Cells were washed once by adding gently 4 ml PBS to the side of the vessel across the attached cell layer, with gentle spinning. Then, PBS was aspirated and 2 ml of Trypsin EDTA was added onto cells, and incubated a few minutes until cells wholly detached from plate surface. 8 ml of fresh pre-warmed medium was added onto the cells to terminate this reaction and the cells were gently pipetted up and down to break the larger clumps. Cells were gathered into a 15 ml fresh tube by micropipette and centrifuged at 300xg for 5 min. Supernatant was removed. Pellet was dispersed in remaining medium by tapping the tube. Complete fresh media was added into the tube and cells were covered into new 100 mm plates at 1:5 ratio.

2.2.22.3 Freezing of HEK293T cell line

When the culture was seen 90% confluent under microscope, cells were detached and transferred into 15 ml centrifuge tube. After centrifugation, cells were re-suspended in 1 ml of Freezing medium and transferred into a cryo tube. Tube was inserted into isopropanol tank and stored at -80°C for short period. Cells should be stored at liquid nitrogen for longer period.

2.2.23 Transfection

2.2.23.1 Transfection of HEK293T cell line with X-tremeGene HP transfection reagent

25×10^4 cells/cm² were plated into culture vessels 24 hours before transfection for 6 well plate. DNA and X-tremeGene HP transfection reagent (Roche) were vortexed before using and equated to room temperature. DNA was diluted in serum free DMEM to get a final concentration of 2 µg DNA/100 µl medium. After that transfection reagent was added directly into the medium containing diluted DNA at 1:3 ratio. Complex were then incubated at room temperature for 15 min. After incubation, complex was added to cultured cells in a dropwise manner. Plate was swirled to ensure distribution of complex over the whole surface of plate. Cells were incubated for 48

hrs with required amounts of DNA, diluent and transfection reagent for 6 well and 100 mm plate were listed in Table 2.19.

Table 2.19: DNA, reagent and media amount for transfection with Xtreme-Gene HP.

Culture vessel	Amount of DNA	Amount of Transfection complex	Amount of Transfection reagent (1:3)
6 well (1 well)	2 µg	200 µl	6 µl
100 mm	10 µg	1000 µl	30 µl

2.2.23.2: Transfection of HEK293T cell line with PEI

Branched and Linear PEI (1mg/ml) Sigma was used for transfection of HEK293T cell line. 24 hours before transfection, 2×10^6 cells/cm² were plated into culture vessels for 100 mm culture dish. Before transfection, medium was removed and plate was washed with 4 ml PBS. After washing step, plate covered with 6 ml antibiotic free medium. For 100 mm culture dish 10 µg DNA was added into 1 ml DMEM free. After that 30 µl PEI reagent added directly into the mix. This mix was incubated 15 min at RT and DNA-PEI mix diluted by adding 1 ml DMEM free. Mixture was added into culture vessel in a drop by drop manner. Plate was shaken to ensure distribution of complex over the whole surface of plate. For Branched PEI reagent; 4 hrs after transfection, antibiotic free medium was removed and plate was washed with 4 ml pre-warmed PBS. After then, 10 ml of fresh medium was added into plate and cells were incubated in a 37°C incubator for 48 hrs with 5% CO₂. Differently Linear PEI reagent not much toxic as Branched reagent. Thus, antibiotic media was aspirated 1 day later transfection and cells were incubated in a 37°C incubator for 48 hrs with 5% CO₂.

3.RESULTS

ATXN3 and MAD2B are two putative NFIB interaction partners that were previously identified as such in a yeast two-hybrid screen. To investigate potential interactions between NFIs and these putative NFIB binding partners, we set out to perform co-immunoprecipitation and GST pull-down assays. To this end ATXN3 and MAD2B were first cloned into appropriate expression vectors.

3.1 Construction of ATXN3 and MAD2B Expression Vectors

To overexpress NFIB's putative interaction partners in mammalian cells, we prepared mammalian and bacterial expression constructs. 56 human alternative splice variants of ATXN3 have been identified so far and these variants can be translated into at least 20 protein isoforms. In these experiments, we used the largest variant which is composed of 376 amino acids with a 22 glutamine-long polyQ stretch at the C terminus. We amplified the open reading frame of this isoform from MSCV-N-FLAG-HA-IRES-PURO (Plasmid #22538, Addgene) retroviral vector and placed this 1,1 kb fragment into the pcDNA3.1 mammalian expression vector by homologous recombination. Similarly, the cDNA of the other putative interaction partner, MAD2B, was from the pGADT7 yeast expression vector and inserted into the pcDNA3.1 with an HA tag on its N-terminus. Once the constructs were verified by DNA sequencing analysis, they were then overexpressed in HEK293T cells to check for expression of ATXN3 and MAD2B proteins. HEK293T cells were transfected with each construct separately and whole cell lysates were analysed by western blotting (Figure 3.1A). Western blot analysis indicated that HA-tagged ATXN3 migrates at ~46 kDa. The molecular weight of HA-ATXN3 was calculated as 44 kDa, however, the tagged protein appeared to be 46 kDa by Western Blot analysis. While we initially used the HA-tag for NFIB interacting partners and His-tagged NFI constructs in co-immunoprecipitation assays, we had to switch tags to control for antibody specificity in the subsequent experiments. To generate an ATXN3 construct with an alternative epitope tag, we amplified ATXN3 from MSCV-N-FLAG-HA-IRES-PURO and inserted this piece into the pcDNA3.1 with a FLAG tag at its N terminus (Saritaş, 2016) producing pcDNA3.1-FLAG-ATXN3. Overexpression of this construct in HEK293T cells leads to a FLAG-tagged protein migrating at ~46 kDa. The molecular

weight of FLAG-ATXN3 was also calculated as 44 kDa, the same as HA-ATXN3 (Figure 3.1B). Using a similar strategy, MAD2B was also cloned into the pcDNA3.1 with a FLAG tag at its N terminus expressing a recombinant protein which migrates at ~26 kDa (Figure 3.1C). Constructs expressing HA-tagged NFIs, pCHNFI-A4, pCHNFI-B2, pCHNFI-C2 and pCHNFI-X2 were gifts from Richard Gronostajski (Chaudhry et al. 1998. Addgene #31404, 31405, 31403, 31402). HA tagged NFIA, NFIB, NFIC and NFIX proteins migrate at ~60 kDa, ~47 kDa, ~58 kDa, ~48 kDa, respectively (Figure 3.4 and Figure 3.5 lower panel).

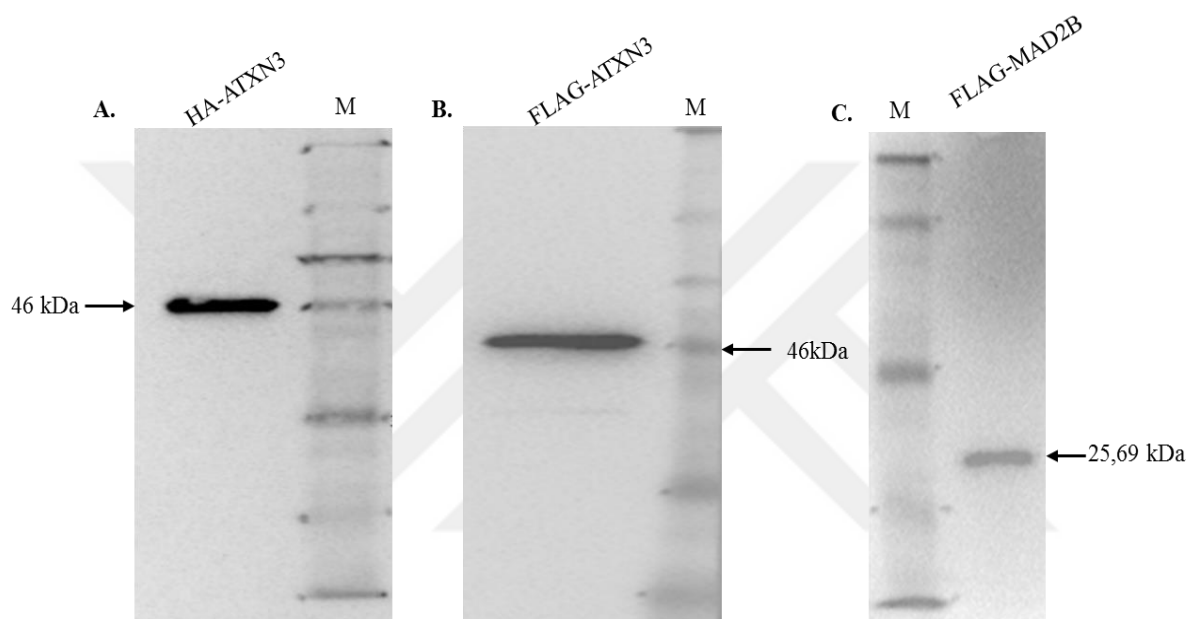


Figure 3.1: Western Bloy analysis of ATXN3 and MAD2B. **A.** Western Blot analysis of HA-ATXN3. Membrane extract from HEK293T cells (10 μ g) was detected using α -HA antibody. The position of HA-ATXN3 indicated with arrow. **B.** Western Blot analysis of FLAG-ATXN3. Membrane extract from HEK293T cells (10 μ g) was detected using α -FLAG antibody. The position of FLAG-ATXN3 indicated with arrow. **C.** Western Blot analysis of FLAG-MAD2B. Membrane extract from HEK293T cells (10 μ g) was detected using α -FLAG antibody. The position of FLAG-MAD2B indicated with arrow. M: Marker.

For GST pull-down experiments, ATXN3 was amplified from pcDNA-FLAG-ATXN3 construct and inserted into the pGEX4T3 bacterial expression vector by homologous recombination. MAD2B was amplified out of pcDNA-HA-MAD2B and inserted into the pGEX4T3 bacterial expression vector. GST-ATXN3 fusion protein (with N-terminal GST), when expressed in bacteria, could be isolated in the soluble fraction of the cell lysates and migrates at ~67 kDa as expected level (Figure 3.2). However, GST-MAD2B protein could not be obtained in the soluble fractions of bacterial lysates and did not migrate at the expected molecular weight of ~52 kDa.

Instead, we observed GST-MAD2B at ~46 kDa and this protein could only be solubilized in the denaturing lysis buffer (Figure 3.17). GST protein alone, which was used as negative control for pull down experiments, migrates at ~26 kDa.

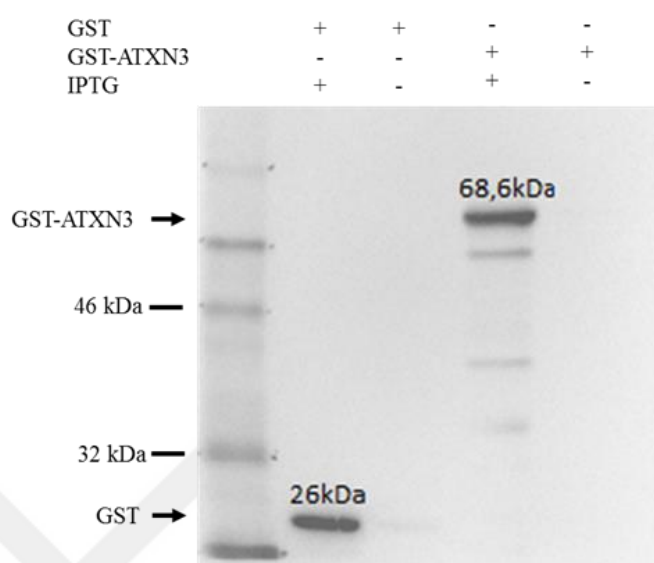


Figure 3.2: Western Blot analysis of GST-ATXN3 and GST. Membrane extracts from HEK293T cells (10 μ g) were detected using α -GST antibody. The positions of GST-ATXN3 and GST indicated with arrows. M: Marker.

3.2 Analysis of NFIs, MAD2B and ATXN3 Interactions by Co-Immunoprecipitation Assays

In order to explore interactions between NFIB and its potential interaction partners MAD2B and ATXN3, we first carried out a co-immunoprecipitation experiment. HEK293T cells were transfected with plasmids that express HA-NFIB together with either FLAG-MAD2B or FLAG-ATXN3. Immune complexes isolated with α -FLAG conjugated agarose resin were subjected to Western Blot analysis using α -HA and α -FLAG antibodies. In this experiment, we see bands corresponding to FLAG-ATXN3 at 46 kDa and FLAG-MAD2B at 26 kDa, indicating these proteins are immunoprecipitated (Figure 3.3, upper panel). However, we also detect additional proteins with mobility similar to ATXN3 and MAD2B in these immune complexes. These proteins which appear at ~50 and 25 kDa could be IgG heavy and light chains originating from α -FLAG resin. To overcome these additional proteins, we replace seconder antibody with the VeriBlot seconder antibody. After that, we always used the same Veri Blot seconder antibody. Moreover, HA-NFIB was found to co-

immunoprecipitate with FLAG-MAD2B (Figure 3.3, lower panel) while little HA-NFIB precipitated from lysates of cells transfected with HA-NFIB only (negative control). Very low levels of HA-NFIB were detected to precipitate with FLAG-ATXN3 when both were co-expressed in HEK293T cells. The amount of NFIB that was precipitated was approximately same as negative control. HEK293T cells transfected with FLAG-ATXN3 and empty vector was also included as another control experiment. FLAG tagged proteins in the starting material (input) was not detected with high sensitivity in this experiment due repetitive use of α -FLAG antibody which was corrected in subsequent experiments.

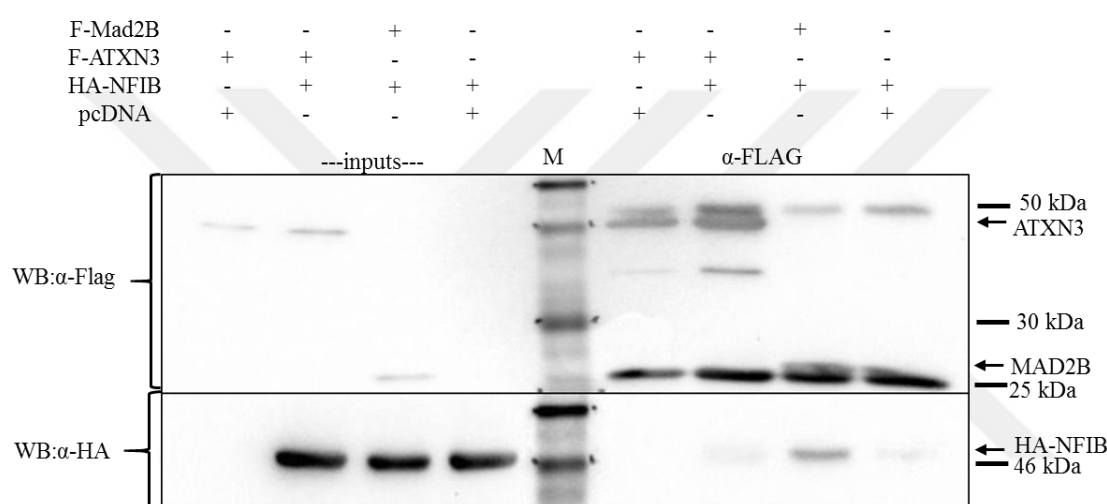


Figure 3.3: Co-immunoprecipitation analysis of HA-tagged NFIB and FLAG-tagged MAD2B and ATXN3. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B and ATXN3 using α -FLAG antibody HA-tagged NFIB using α -HA antibody. The position of NFIB immunoreactive band, ATXN3 and MAD2B is indicated with arrow. Immunoprecipitates are shown together with 10 μ g of each input lysate. The position of NFIB, ATXN3 and MAD2B immunoreactive bands are indicated with arrows. IgG heavy and light chains originating from α -FLAG resin are indicated with bars correspond to 50 kDa and 25 kDa respectively. M:Marker

Next we repeated this co-immunoprecipitation experiment, incorporating additional controls. HA-NFIB was co-transfected with either FLAG-MAD2B, or FLAG-ATXN3, as before. However, in addition, FLAG-MAD2 was included as a negative control; MAD2B is 23% identical and 54% similar to mitotic arrest-deficient 2 (MAD2) at the amino acid level and is named after this protein. Moreover, FLAG-PIN1, which has previously been found to interact with NFIB (Saritaş, 2016), was used as positive control. We precipitated FLAG-MAD2B as well as FLAG-MAD2 albeit in

lower amounts (Figure 3.4). Levels of FLAG-MAD2 in the starting material was lower than that of other FLAG tagged proteins suggesting that transfection of, or expression from this plasmid is not as efficient. Levels of ATXN3 in α -FLAG immune complexes was similar to MAD2B levels. As expected, HA-NFIB does co-precipitate with FLAG-PIN1. However, the appearance of unexpected additional bands around 50 kDa confounds this particular result. This approximately 50kDa protein, indicated with an asterisk (Figure 3.4, upper panel) could correspond to IgG heavy chains. HA-NFIB was also found to co-precipitate with FLAG-MAD2B, in lower amounts compared to PIN1 containing immune complexes. The amount of HA-NFIB that was co-precipitated could be correlated with the levels of FLAG-tagged protein in the immune complexes (compare HA immunoreactivity in MAD2B and PIN1 lanes). Moreover, NFIB could not be immunoprecipitated with ATXN3, consistent with the previous co-immunoprecipitation result obtained with α -FLAG conjugated resin (Figure 3.3).

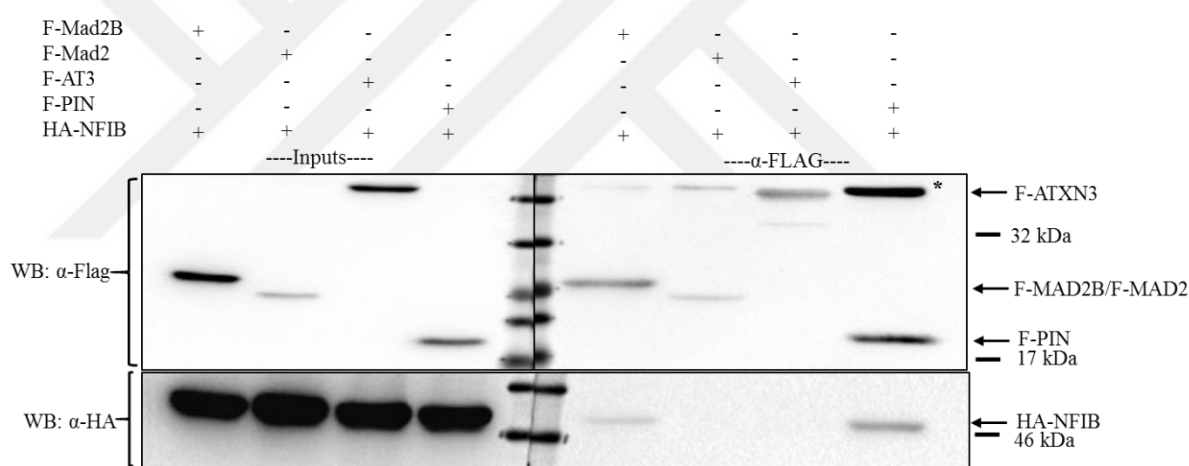


Figure 3.4: Co-immunoprecipitation analysis of HA-tagged NFIB and FLAG-tagged MAD2B, ATXN3, MAD2 as negative control and PIN as positive control.

Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B, ATXN3, MAD2 and PIN using α -FLAG antibody and HA tagged NFIB using α -HA antibody, followed by western blot analysis of HA-tagged NFIB. Immunoprecipitates are shown together with 10 μ g of each input lysate. The position of NFIB, ATXN3, MAD2B, MAD2 and PIN immunoreactive bands are indicated with arrows. IgG heavy chains illustrated with star is shown in the figure upper panel. M: Marker.

Next, we wanted to find out if other NFI family members could interact with MAD2B. We performed co-immunoprecipitation experiments overexpressing FLAG-MAD2B with HA tagged NFIA, NFIC, NFIX or an NFIB mutant form that contains only the C-terminal domain (NFIB_C). (As explained below, we did not initially use constructs that express only the NFIB N-terminal domain as expression levels are low.) First of

all, FLAG-MAD2B expression levels in the starting material varies between transfection experiments, with MAD2B levels being lowest in NFIX cotransfection, leading to different levels of FLAG-MAD2B to be precipitated (Figure 3.5, upper panel). However, no HA immunoreactive bands are observed regardless of expression levels or the amount of MAD2B in immune complexes (Figure 3.5, lower panel). This finding suggests that MAD2B does not bind NFIA or NFIX as strongly as NFIB, and the interaction does not involve the NFIB C-terminal. However, HA-NFIB was co-precipitate with both FLAG-MAD2B and FLAG-PIN (Figure 3.4 lower panel). These experiments results shown at Figure 3.4 and Figure 3.5, performed in same blot. We have repeated this experiment and obtained similar results (Appendix E).

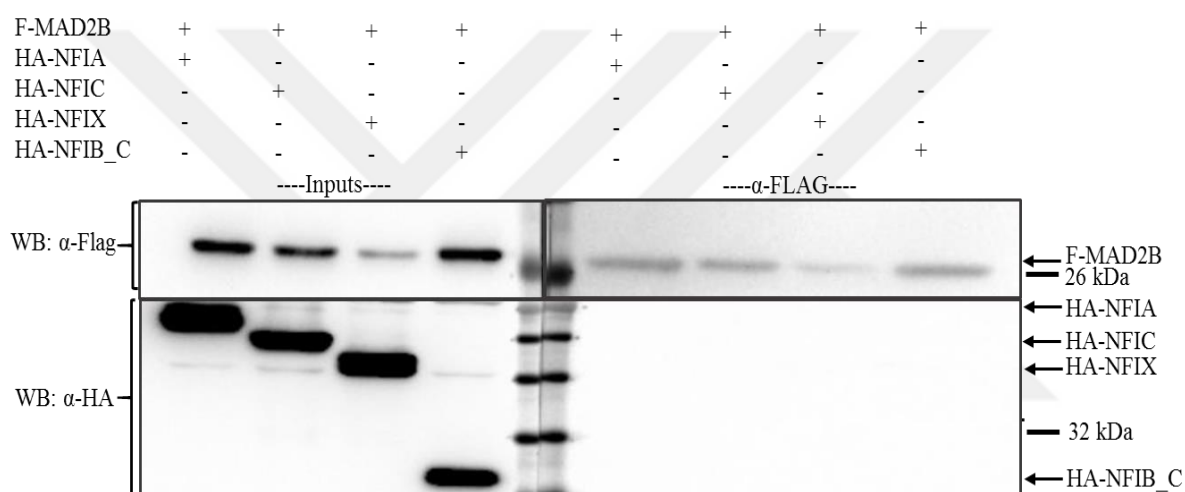


Figure 3.5: Co-immunoprecipitation analysis of HA-tagged NFIA, NFIC, NFIX and NFIB_C and FLAG-tagged MAD2B. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B using α -FLAG antibody and HA tagged NFIs using α -HA antibody, followed by western blot analysis of HA-tagged NFIs and FLAG tagged MAD2B. Immunoprecipitates are shown together with 10 μ g of each input lysate. The position of NFIA, NFIC, NFIX, NFIB_C and MAD2B immunoreactive bands are indicated with arrows. M: Marker.

We thus found that the NFIB C-terminal domain may not be involved in NFIB interactions with MAD2B. In order to test if MAD2B actually binds the NFIB N-terminal DNA binding and dimerization domain in these assays, co-immunoprecipitation experiments were performed using lysates of HEK293T cells overexpressing FLAG-MAD2B with NFIB dominant negative mutant constructs, NFIB_N or NFIB_E. NFIB_N contains only the N-terminal domain while NFIB_E codes for NFIB DNA-binding domain (DBD) fused to the engrailed repressor. Both constructs were previously generated (Güler, 2016; Bachurski 2003, Namihira 2009,

Wang 2004). We again observed variations in levels of MAD2B expression (Figure 3.6 upper panel). Moreover, amount of FLAG-MAD2B that was immunoprecipitated also varied, with lower amounts being precipitated in the NFIB_N co-immunoprecipitation assay. Levels of NFIB_N and NFIB_E proteins were also significantly lower in the starting material. While we did not detect NFIB_N, NFIB_E or NFIB_C with FLAG-MAD2B in the precipitated immune complexes, we cannot definitively interpret these findings as the NFIB_N and NFIB_E protein expression levels were quite low. However, the C-terminal domain of NFIB does not appear to interact with MAD2B on its own in these experiments (Figure 3.6 lower panel). Interestingly, MAD2B did not precipitate at expected levels (Figure 3.6, indicated with an asterisk) even though we did observe HA-NFIB in these immune complexes (Figure 3.6, upper panel).

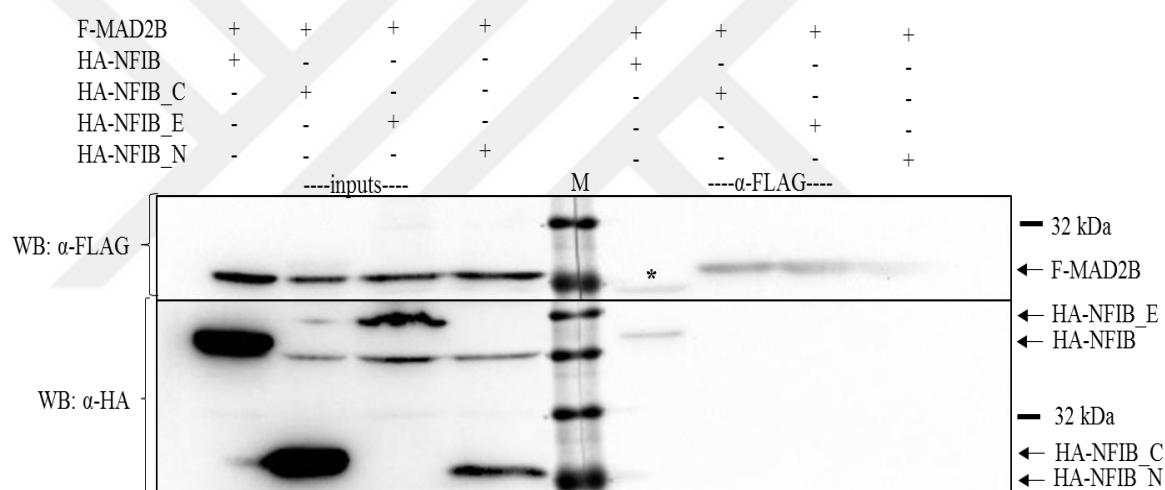


Figure 3.6: Co-immunoprecipitation analysis of HA-tagged NFIB, NFIB_C, NFIB_N and NFIB_E with FLAG-tagged MAD2B. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B using α -FLAG antibody and HA tagged NFIB and mutant forms using α -HA antibody, followed by western blot analysis of HA-tagged NFIB, NFIB_C, NFIB_N and NFIB_E and FLAG tagged MAD2B. Immunoprecipitates are shown together with 10 μ g of NFIB and NFIB_C input lysate and 30 μ g of NFIB_N and NFIB_E input lysates. The position of NFIB, NFIB_C, NFIB_N, NFIB_E and MAD2B immunoreactive bands are indicated with arrows. M:Marker.

Finally, we repeated α -FLAG immunoprecipitation experiments including additional controls. We contranfect HEK293T cells with these combinations of constructs: MAD2B-NFIB, MAD2B-pcDNA, NFIB-pcDNA or MAD2-NFIB. Once again, FLAG tagged MAD2B and MAD2 and HA tagged NFIB was expressed. Additionally, we included empty vector (pcDNA) as negative control, to check for nonspecific

binding HA-NFIB to α -FLAG resin and presence of nonspecific bands in immune complexes isolated from lysates containing MAD2B alone. According to this experiment NFIB precipitated with FLAG-MAD2B, but we could not observe an α -HA immunoreactive band (representing HA-NFIB) in NFIB-pcDNA lysates or in FLAG-MAD2B-pcDNA lysates (Figure 3.7, lower panel). Additionally we have encountered one problem in this experiment, levels of FLAG-MAD2 in the starting material is lower than that of other FLAG tagged proteins suggesting that transfection of, or expression from this plasmid is not as efficient (Figure 3.7, upper panel). Thus, MAD2 band might shown less on membrane immunoprecipitated side compared to MAD2B (Figure 3.7, upper panel). Overall, α -FLAG co-immunoprecipitation results indicate that NFIB precipitated with MAD2B just like PIN. However, we could not observe NFIB to precipitate with ATXN3 or MAD2 in any of the co-immunoprecipitation experiments.

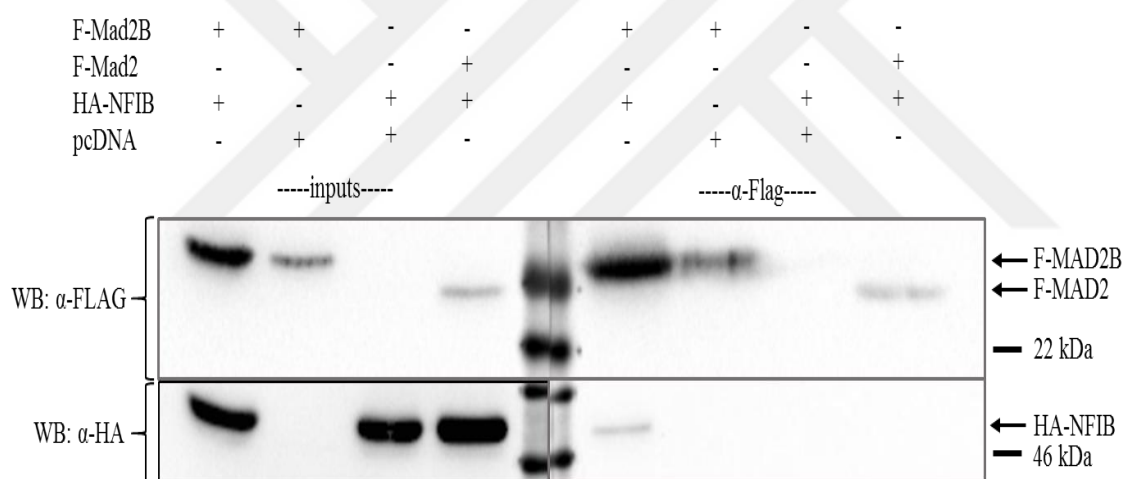


Figure 3.7: Co-immunoprecipitation analysis of HA tagged NFIB and FLAG tagged MAD2B and MAD2. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B and MAD2 using α -FLAG antibody and HA tagged NFIB using α -HA antibody, followed by western blot analysis of HA-tagged NFIB and FLAG tagged MAD2B and MAD2. Immunoprecipitates are shown together with 10 μ g of each input lysate. The position of NFIB, MAD2B and MAD2 immunoreactive bands are indicated with arrows.
M:Marker.

Next, we performed co-immunoprecipitation experiments with α -HA conjugated agarose resin, to test whether MAD2B in turn would immunoprecipitate with HA-NFIB and other NFI family members. First of all, FLAG-MAD2B, FLAG-MAD2, FLAG-ATXN3 or FLAG-PIN1 were co-expressed with HA-NFIB as before. In this experiment, we again see some variability in the expression levels of these exogenous

proteins when co-expressed (Figure 3.8). There is also some variability in the amounts of NFIB that was precipitated, with higher levels detected in assays with MAD2 and ATXN3. (The activity of the secondary antibodies was probably the reason for apparent low sensitivity of detection in this blot.) While levels of HA-NFIB was the lowest (Figure 3.8, upper panel) in MAD2B and PIN1 co-immunoprecipitation experiments, we nevertheless were able to determine that both proteins co-immunoprecipitate with NFIB. We detected no ATXN3 or MAD2 in HA-NFIB precipitates; similar results were obtained when the experiment was repeated (Appendix E). As FLAG-MAD2 overexpression is quite low (Figure 3.8, lower panel), it is difficult assay for interactions of this protein with NFIB, however, MAD2B does appear to interact with NFIB based on this assay.

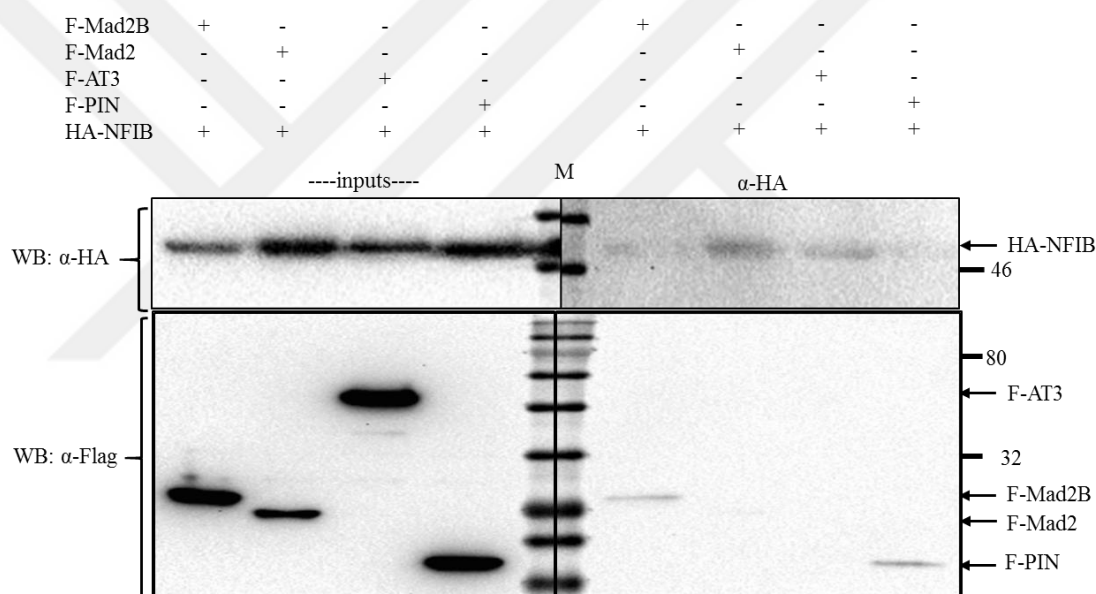


Figure 3.8: Co-immunoprecipitation analysis of HA-tagged NFIB and FLAG-tagged MAD2B, ATXN3, MAD2 as negative control and PIN as positive control.

Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B, ATXN3, MAD2 and PIN using α -FLAG antibody and HA-tagged NFIB using α -HA antibody, followed by western blot analysis of HA-tagged NFIB and FLAG tagged MAD2B, ATXN3, PIN and MAD2. Immunoprecipitates are shown together with 10 μ g of each input lysate. The position of NFIB, ATXN3, MAD2, MAD2B and PIN immunoreactive bands are indicated with arrows. M: Marker

In parallel experiments, we tested for binding to HA-NFI family members and NFIB_C using the same approach, immunoprecipitating NFIs with α -HA conjugated agarose resin. Once again, while NFIA, NFIC, NFIX and NFIB_C were all efficiently expressed and immunoprecipitated (Figure 3.9, upper panel), no MAD2B co-

precipitated with any of these proteins, suggesting that NFIs do not interact with MAD2B under these *in vitro* conditions (Figure 3.9, lower panel). These results are consistent with those obtained with co-immunoprecipitation experiments using α -FLAG resin (Figure 3.4 and Figure 3.5).

In short, co-immunoprecipitation using α -HA conjugated agarose resin and α -FLAG conjugated agarose resin showed that FLAG-MAD2B is precipitated with HA-NFIB and vice versa. However, there was no indication for MAD2B binding to NFIA, NFIC, or NFIX. Moreover, while we were unable to convincingly test for binding to NFIB DBD, the NFIB C-terminal domain also did not exhibit any affinity for MAD2B in these experiments. Finally, ATXN3 protein did not interact with NFIB protein in either of these co-immunoprecipitation experiments.

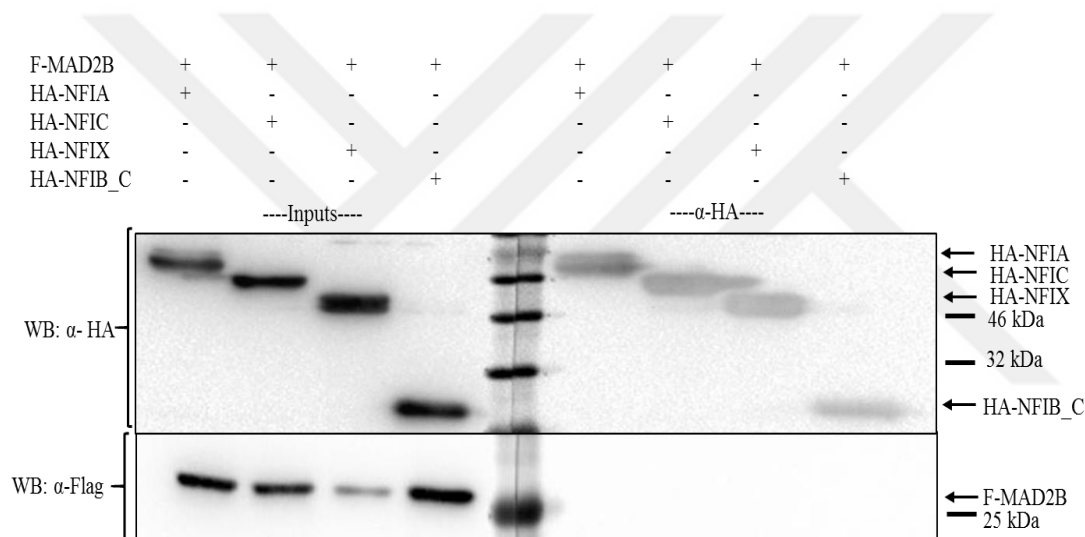


Figure 3.9: Co-immunoprecipitation analysis of HA-tagged NFIA, NFIC, NFIX and NFIB_C and FLAG-tagged MAD2B. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of FLAG tagged MAD2B using α -FLAG antibody and HA-tagged NFIs using α -HA antibody, followed by western blot analysis of HA-tagged NFIs and FLAG tagged MAD2B. Immunoprecipitates are shown together with 10 μ g of each input lysate. The position of NFIA, NFIC, NFIX, NFIB_C and MAD2B immunoreactive bands are indicated with arrows. M: Marker

3.3 Analysis of NFIs and ATXN3 Interactions by GST-Pull Down Assays

As seen above, ATXN3 did not co-immunoprecipitate with NFIB and NFIB did not co-immunoprecipitate with ATXN3. We also assessed binding of these proteins using an alternative approach, the Glutathione S transferase (GST) pull down assay. To this end, first we generated an expression plasmid that encodes the GST-ATXN3 fusion protein. Then, expression of both Glutathione S transferase (GST) and GST-ATXN3 was induced in BL21 *E. coli*. This strain allows high-levels protein expression when

transformed with a gene that is under the control of T7 promoter. First we checked for expression of GST-ATXN3 fusion protein after inducing the cells with IPTG for two hours. Both GST and GST-ATXN3 were detected in the soluble fraction of BL21 cell lysates (Figure 3.10). While GST-ATXN3 protein migrates at ~68 kDa, GST protein alone migrates at ~26 kDa and was used as negative control for pull down experiments.

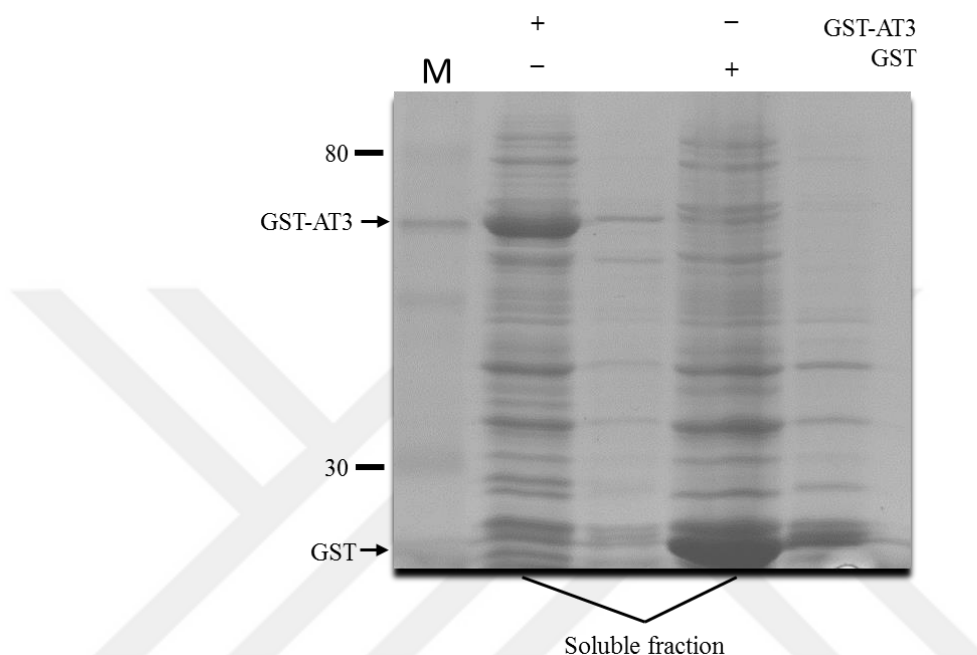


Figure 3.10: Analysis of GST-ATXN3 and GST by SDS-PAGE. GST-ATXN3 is in the soluble fraction of BL21 cells. BL21 cells were transformed with plasmids expressing GST or GST-ATXN3, induced with IPTG for 2 hr and lysed using BugBuster. Insoluble fractions were resuspended with 1X Sample Buffer. Soluble fractions were resolved by 10% SDS-PAGE. Protein bands which correspond to GST and GST-ATXN3 were visualized by coomassie blue staining. Empty lanes between samples exhibit pellet of examples, not contain GST and GST-ATXN3 protein. Bands correspond to GST and GST-ATXN3 are indicated with arrows. M: Marker.

Next, the soluble extracts containing GST or GST-ATXN3 were added to and GST or GST-ATXN3 were bound to Glutathione Sepharose resin. Membrane extracts of HEK293T cells overexpressing HA-NFIB were then incubated with GST or GST-ATXN3 bound resin. When we analysed proteins that were “pulled-down” by GST or GST-ATXN3, we saw that equivalent amount of GST and GST-ATXN3 were isolated with glutathione resin (Figure 3.11, upper panel). However, while GST-ATXN3 bound HA-NFIB, GST did not bind to NFIB to the same extent (Figure 3.11, lower panel).

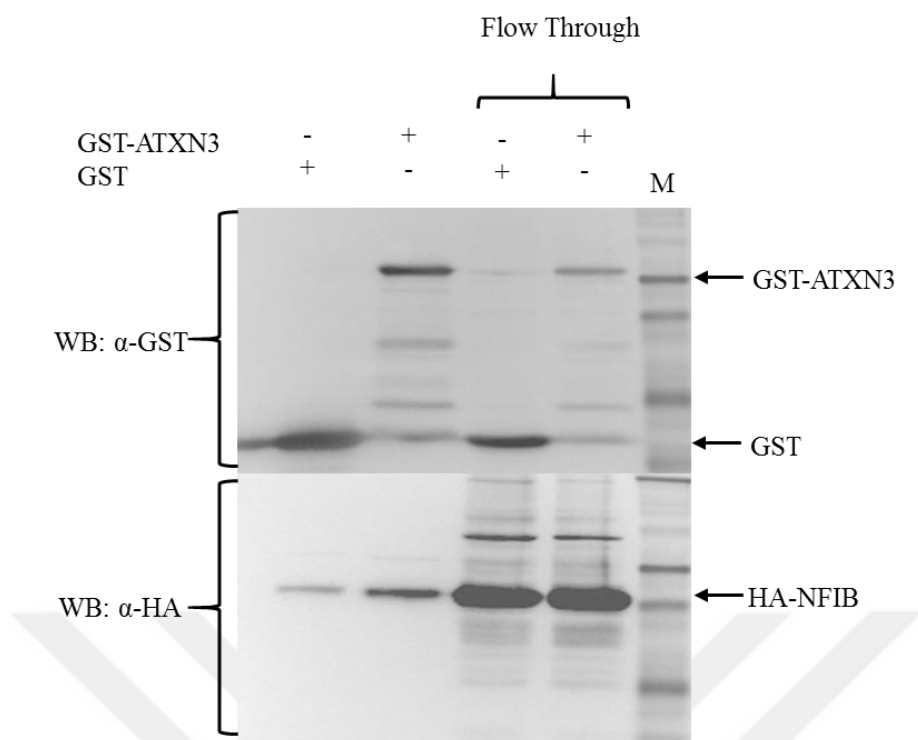


Figure 3.11: GST Pull down analysis of HA-tagged NFIB with GST-ATXN3 and GST. 100µg HEK293T lysate overexpressed HA-tagged NFIB was incubated with bacterially expressed GST or GST-ATXN3 immobilized on glutathione sepharose resin. After centrifugation, pellets were eluted in 1X Sample Buffer. 10% of supernatant were loaded as flow-through (F.T) that exhibit not bound HEK293T lysate on resin. Eluates of HA-tagged NFIB incubated with GST (lane 1), incubated with GST-ATXN3 (lane 2) and flow through of HA-tagged NFIB incubated with GST and GST-ATXN3 (lane 3, lane 4) are shown. Immunoreactive bands correspond to HA tagged NFIB, GST and GST-ATXN3 are indicated with arrows. M: Marker.

Next, we wanted to compare levels of NFIB binding to ATXN3 with dominant negative NFIB constructs. To this end, HEK293T lysates overexpressing NFIB, NFIB_N or NFIB_E were incubated with either GST or GST-ATXN3. In this experiment, levels of NFIB, NFIB_N and NFIB_E expression varied. In order to use comparable amounts of exogenous protein in these experiments, expression levels in all lysates were first checked via western blot analysis. In this experiment, approximately 100 µg protein lysate was used for incubation with either GST or GST-ATXN3. Levels of NFIB_N were quite low in the starting material and NFIB_E was not detected at all (Figure 3.12). However, we found that both proteins are pulled down by ATXN3 in this experiment. Surprisingly, NFIB itself could be pulled down by both GST and GST-ATXN3. ATXN3 appeared to interact with NFIB, as well as NFIB_E and NFIB_N moderately (Figure 3.12).

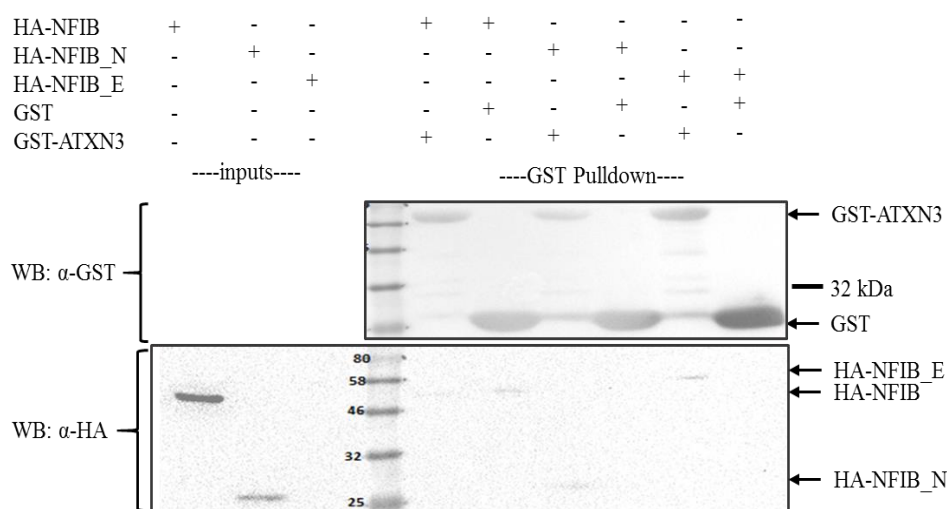


Figure 3.12: GST Pulldown analysis of HA-tagged NFIB, NFIB_E and NFIB_N with GST-ATXN. 10 μ g GST-ATXN3 and 15 μ g GST were immobilized on resin. 100 μ g HEK293T lysate overexpressing HA-tagged NFIB, HA-tagged NFIB_N or HA-tagged NFIB_E was incubated with bacterially expressed GST or GST-ATXN3 immobilized on glutathione sepharose resin. Bands correspond to GST, GST-ATXN3, NFIB, NFIB_N and NFIB_E indicated with arrows.

In order to verify these findings, we performed another GST pulldown experiment with NFIB_N and NFIB_E proteins. This time, we achieved higher levels of both proteins in the starting material (Figure 3.13). While we see some background bands for NFIB_E, both mutant NFIB proteins, which carry the NFIB DBD, seem to be pulled down by GST-ATXN3, but not by GST (Figure 3.13). These data suggest that NFIB N-terminal DNA binding and dimerization domain could be sufficient to interact with ATXN3 alone.

To assay for binding of the other NFI family members to ATXN3, each NFI was overexpressed and incubated with GST or GST-ATXN3. We used similar amount of overexpressed protein lysates as 100 μ g. Interestingly, even though HA-NFIs are expressed from the same vector backbone, levels of expression vary significantly. Equivalent amount of HEK293T overexpressed protein lysates used for incubation with either GST or GST-ATXN3. Nevertheless, ATXN3 appeared to interact with HA-NFIA and HA-NFIB (Figure 3.14). However, NFIC, NFIX and NFIB_C binding to ATXN3 was not observed (Figure 3.15). Similar results were obtained when the experiment was repeated (Appendix E).

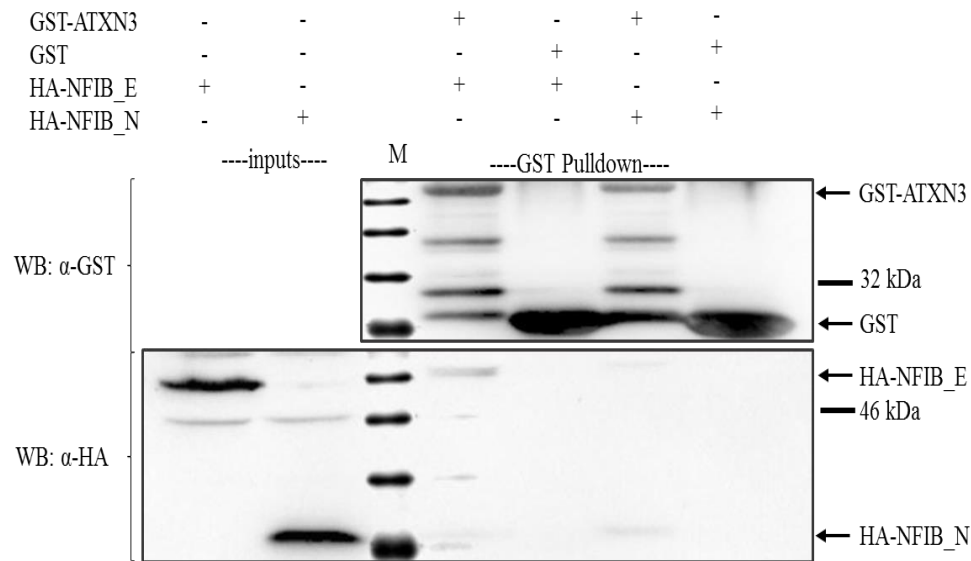


Figure 3.13: GST Pulldown experiment analysis of HA-tagged NFIB_E and NFIB_N with GST-ATXN3. Input lysates of NFIs used in pulldown experiment. Approximately 10 μ g GST-ATXN3 and 15 μ g GST were immobilized on resin. Equivalent amount of NFI lysates (100 μ g) were used for pull down with GST-ATXN3. Bands correspond to GST, GST-ATXN3, NFIB, NFIB_T and NFIB_E indicated with arrows. M: Marker.

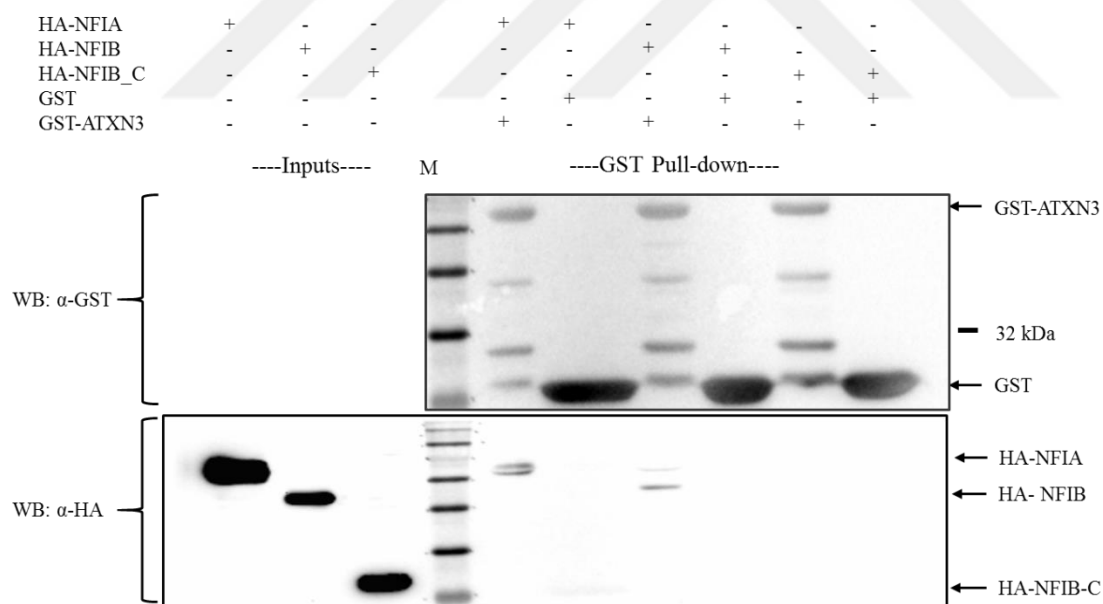


Figure 3.14: GST pull down analysis HA-tagged NFIA, NFIB, NFIB_C with GST-ATXN3 and GST. 100 μ g HEK293T lysate overexpressing HA-tagged NFIA, HA-tagged NFIB or HA-tagged NFIB_C was incubated with bacterially expressed GST or GST-ATXN3 immobilized on glutathione sepharose resin. Approximately 10 μ g GST-ATXN3 and 15 μ g GST were immobilized on resin. Black line indicate marker on the right side. Bands correspond to GST, GST-ATXN3, NFIA, NFIB and NFIB_N indicated with arrows. M: Marker.

Thus, GST pulldown experiments suggest that ATXN3 may bind to NFIA, NFIB, NFIB_N and NFIB_E. However, we have not detected ATXN3 binding to the NFIB C terminal domain. Further experiments will have to be performed to identify which NFIB domains or regions interact with ATXN3.

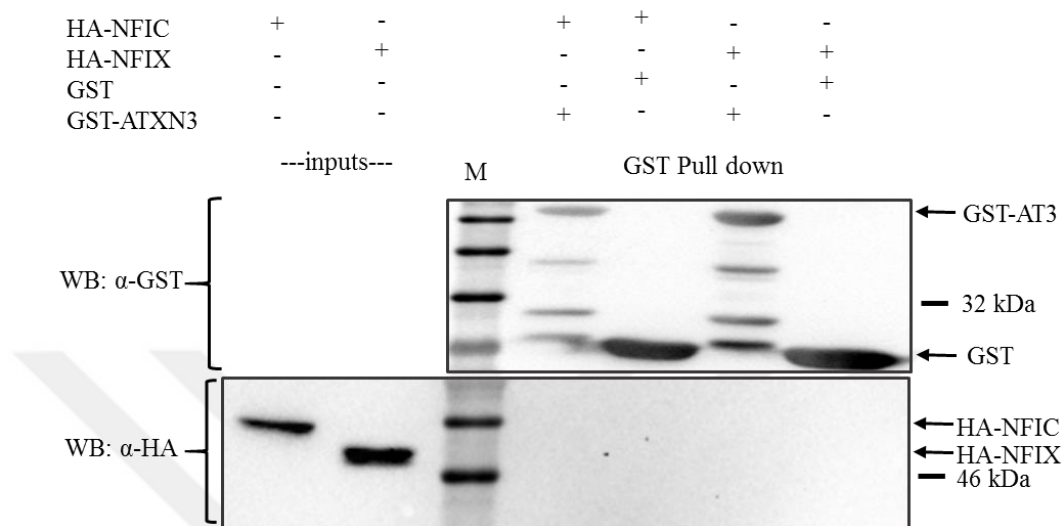


Figure 3.15: GST pull down analysis HA-tagged NFIC, NFIX and GST-ATXN3. 100 µg HEK293T lysate overexpressing HA-tagged NFIC or HA-tagged NFIX was incubated with bacterially expressed GST or GST-ATXN3 immobilized on glutathione sepharose resin. Approximately 10 µg GST-ATXN3 and 15 µg GST were immobilized on resin. Bands correspond to GST, GST-ATXN3, NFIC and NFIX indicated with arrows. M: Marker.

As we did see an interaction between MAD2B and NFIB using the co-immunoprecipitation experiments, we also wanted to confirm this finding using the GST pulldown assay. To this end, we first set out to express the GST-MAD2B fusion protein in *E. coli*. Expression of both Glutathione S transferase (GST) and GST-MAD2B was induced in BL21 and Rosetta *E. coli*, strains suitable for high-level protein expression of transformed gene under the control of T7 promoter. First we checked for expression of GST-MAD2B fusion protein after two hour of induction with IPTG. We analysed GST-MAD2B and GST expression by Western Blot in two different cell types, Rosetta and BL21. However, GST-tagged MAD2B protein could not be obtained in the soluble fraction and did not migrate at the expected molecular weight at ~52 kDa, in either strain. We observed GST-MAD2B at ~46 kDa and not soluble (Figure 3.16). GST protein alone was used as negative control as described above. Thus, as we were unable to solubilize GST-MAD2B with the correct expected size, we were unable to proceed with these experiments.

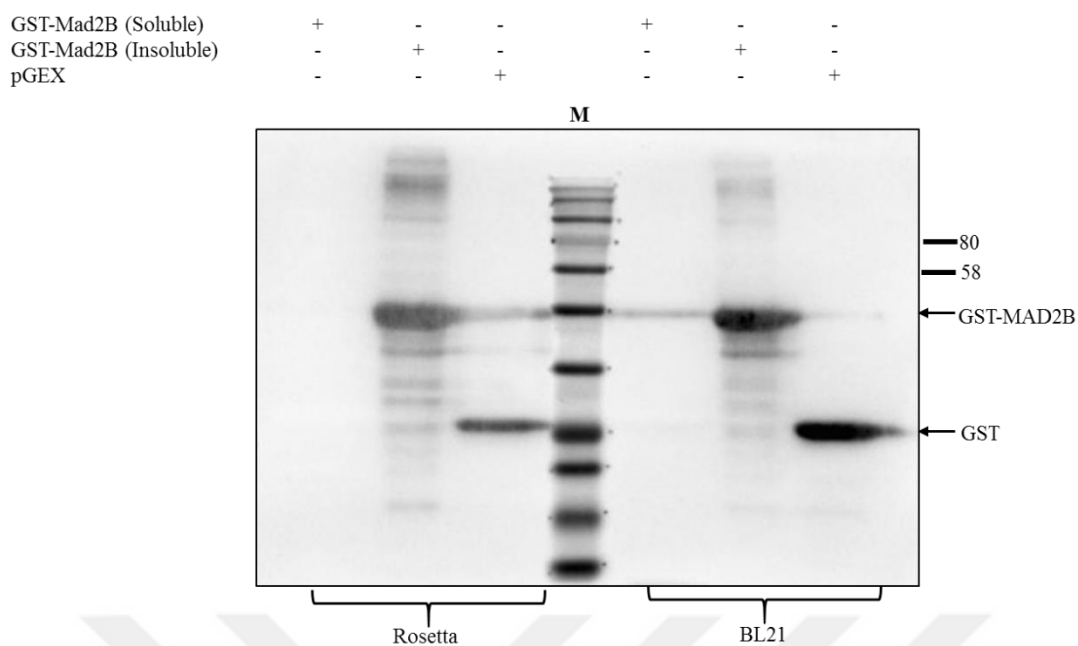


Figure 3.16: Analysis of GST-MAD2B and GST by Western Blot with two different cell types, Rosetta and BL21. Cells were transformed with plasmids expressing GST or GST-MAD2B, induced with IPTG for 2 hr and lysed using BugBuster. Insoluble fractions were resuspended with 1X Sample Buffer. Soluble fractions and pellets were resolved by 10% SDS-PAGE. Protein bands which correspond to GST and GST-MAD2B visualized via α - GST antibody. Empty lanes, head of the samples exhibit soluble part, not contain GST and GST-MAD2B protein. Bands correspond to GST and GST-MAD2B indicated with arrows. M: Marker

4. DISCUSSION AND CONCLUSION

In this thesis, we investigated interactions between NFIs and putative binding partners ATXN3 and MAD2B via co-immunoprecipitation and GST pull down assays. MAD2B is an important intermediary of the spindle assembly checkpoint (SAC). MAD2B appears to function by binding to a diverse spectrum of proteins via its conserved HORMA domain (Aravind et al., 1998). HORMA domain is a multifunctional protein-protein interaction module located in different eukaryotic signaling pathways including the spindle assembly checkpoint, DNA recombination/repair pathways and the initiation of autophagy. This conserved domain binds a sequence is denoted as $\phi\phi xPxxxpP$ (where ϕ is an aliphatic residue, x is any amino acid residue, P and p designate proline, where at the eighth position is less important for interaction) (Hanafusa et al., 2015). Interestingly, NFIB does not appear to carry this motif.

Co-immunoprecipitation experiments show that MAD2B interacts with NFIB when they are co-expressed in HEK293T cells. Moreover, we performed reciprocal co-immunoprecipitation experiments with α -FLAG and α -HA agarose resin to explore MAD2B and NFIB interactions. In these experiments, FLAG-MAD2B could precipitate HA-NFIB and HA-NFIB, in turn, could precipitate FLAG-MAD2B. However, MAD2B interaction with other NFI members (NFIA, NFIC and NFIX) was not observed in either co-immunoprecipitation assay using α -HA or α -FLAG agarose resin. Moreover, we assayed for binding to the NFIB mutants, that contained the N terminal, NFIB_N and NFIB_E, and NFIB C-terminal domain, NFIB_C, precipitating FLAG-MAD2B with α -FLAG antibodies. However, MAD2B could not co-precipitate with NFIB_C, NFIB_N and NFIB_E. Neither could NFIB_C precipitate NFIB. As expression levels of NFIB N-terminal domain mutants were quite low, these results were inconclusive. However, MAD2B appeared not to bind to the NFIB C-terminal region in these experiments. These data suggest that MAD2B may interact with NFIB and that the C-terminal domain is not sufficient to maintain this interaction.

Previously, MAD2B has been shown to interact with transcription factors such as TCF4, and Sim2, which both play roles in developmental processes (Hong et al., 2009, Meng et al., 2012). Knock-out studies show that MAD2B plays a regulatory role in primordial germ cells through modulation of their epigenetic programming (Pirouz et

al., 2013). Furthermore, MAD2B null ESCs are unstable and are restricted in their developmental potential (Pirouz et al., 2015). MAD2B affects both FGF and Wnt signaling via functional binding to the transcription factors Elk-1 and TCF-4 (Zang et al., 2007; Hong et al., 2009) and MAD2B is mainly expressed in neurons of the cortex and hippocampus, which suggests that MAD2B may play a regulatory role in neurons (Meng et al., 2012).

As these experiments test for interactions *in vitro*, using overexpressed tagged proteins, further experiments need to be performed to validate MAD2B-NFIB interactions. First of all, co-immunoprecipitation experiments can be performed using tissue extracts, assaying for interactions of endogenous proteins. Next, site directed mutagenesis can be employed to alter potential binding domains in NFIB or MAD2B. Mutating residues in the N-terminal region or C-terminal region of NFIB may alter its affinity for MAD2B. For example, NFIB N-terminal DNA-binding/dimerization domain contains four cysteine residues and strongly conserved among NFIs. These mutants can be assayed for binding MAD2B via co-immunoprecipitation experiments. Similarly, MAD2B protein interaction domain, the HORMA domain could be mutated and mutants could be tested for their ability to co-precipitate with NFIB.

As these experiments test for interactions *in vitro*, using overexpressed tagged proteins, further experiments need to be performed to validate MAD2B-NFIB interactions. First of all, co-immunoprecipitation experiments can be performed using tissue extracts, assaying for interactions of endogenous proteins. Next, site directed mutagenesis can be employed to alter potential binding domains in NFIB or MAD2B. For example, NFIB N-terminal DNA-binding/dimerization domain contains four cysteine residues that are strongly conserved among NFIs. Mutating highly conserved residues in the N-terminal region or C-terminal region of NFIB may alter its affinity for MAD2B. These mutants can be assayed for binding MAD2B via co-immunoprecipitation experiments. Similarly, MAD2B protein interaction domain, the HORMA domain could be mutated and mutants could be tested for their ability to co-precipitate with NFIB.

Another approach would involve domain swapping between NFIB and NFIs. In co-immunoprecipitation experiments only NFIB showed affinity for MAD2B. In order to pinpoint the interacting domain on NFIB, we could first try swapping the NFIB N-terminal and C terminal domains with another NFI family member. Since neither NFIB

N-terminal domain or C-terminal domain alone seems to interact with MAD2B, we surmised that full length NFIB protein is required for the interaction. NFI N-terminal domain is highly conserved between the four members of NFI family, while the C-terminal domain is varied (Mason et al., 2008). NFIB and NFIA N-terminal domains are 87.4% similar, somewhat higher than other NFIs (NFIB_NFIC: 87% and NFIB_NFIX: 85%). Chimeric fusion proteins which contain the N-terminal domain of NFIB and the C-terminal domain of NFIX or vice versa could be created by domain swapping. Previously, Chaudhry, et al, (1998) has already designed such chimeric fusion proteins with NFIX and NFIB. They swapped the N-terminal regions, creating the chimeric fusion constructs pCHNFI-B/X and pCHNFI-X/B. Similar chimeric constructs can be further investigated for their ability to bind MAD2B. The advantage of this approach is that, these chimeric proteins would presumably be more stable and fold correctly in comparison to constructs that only contain the NFIB N terminal or C terminal domains (NFIB_N and NFIB_C) used in this study.

As part of this study, we also performed GST pulldown assays with NFIs and ATXN3. ATXN3 belongs to the family of polyglutamine proteins and consists of two major domains. C-terminal domain of ATXN3 includes a polyglutamine tract and the N-terminus comprises the Josephin domain which has deubiquitination activity. Flexible C-terminal tail of ATXN3 contains 2 or 3 ubiquitin interacting motifs (UIMS) and a polyQ sequence of variable length. ATXN3's UIMs contain 5 serine residues that are phosphorylated, while the Josephin domain includes one lysine residue that can be ubiquitylated.

In the GST pulldown experiments, NFIB and NFIA were pulled down with GST-ATXN3. Other NFI family members, NFIC and NFIX could not be precipitated by bacterially expressed GST-ATXN3. To find the NFIB region that is involved in this interaction, we used mutant NFIB proteins which either contain only the NFIB C-terminal domain (NFIB_C) or the N-terminal DNA binding domain (NFIB_N and NFIB_E). Preliminary data indicate that GST-ATXN3 is pulled down with NFIB_N and NFIB_E but not with NFIB_C. These data suggest that NFIB N-terminal region is necessary for NFIB-ATXN3 interactions but the C-terminal region alone is not sufficient for binding. Interestingly, if ATXN3 does bind to NFIB and NFIA, it could be to ~~through~~ their N-terminal regions which are 87.4 % similar as described above.

In this study, we used NFI overexpressing HEK293T cell lysates and bacterially overexpressed ATXN3, and assayed for interaction *in vitro*. To verify these results, we need to employ alternative approaches. To test whether NFI family members and NFIB binding partners directly bind each other, we can also use a cell-free translation system which enables us to explore direct associations between cell-free translated NFIs and bacterially expressed interaction partners. ATXN3 appears to preferentially bind NFIA and NFIB, a finding that needs further examination. As described for MAD2B-NFIB interactions, we can perform domain swapping experiments for these two proteins. Chimeric proteins which consist of the N-terminal domain of NFIA or NFIB and C-terminal domain of NFIX can be used to test for binding to ATXN3. Along with these experiments, mutating specific domains of ATXN3, NFIB and NFIA might change binding affinity and reveal which of these domains is essential for the interaction. These additional experiments could corroborate findings of the GST pull down assays conducted in this study.

Both ATXN3 and MAD2B proteins are abundantly expressed in brain regions where NFIs are also present. MAD2B is predominantly expressed in neurons of the cortex and hippocampus (Meng et al., 2012), suggesting that MAD2B may regulate cellular processes in neurons. ATXN3 is generally a cytoplasmic protein, widely expressed in neural and non-neural human tissues. ATXN3 is expressed throughout the human brain in specific neuronal subpopulations. Immunohistochemical studies show that in neurons, ATXN3 is localized in the cytoplasm, proximal processes, and axons and rarely in the nucleus suggesting that regulation of ATXN3 expression levels and its cellular localization may be functionally important (Trottier et al., 1998). Knock-out studies in mice show that both MAD2B and ATXN3 are important key factors controlling developmental and neurological processes (Meng et al., 2012, Tsou et al., 2013). Moreover, ATXN3 and MAD2B both interact with and regulate transcription factors and could similarly regulate NFIB. Therefore, it will be important to verify the preliminary data on binding *in vitro* and further explore whether ATXN3 and MAD2B actually affect NFIB function *in vivo*.

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APPENDICES

Appendix A: Cehimicals and Commercial kits used in this research listed with Supplier companies.

Appendix B: Equipments used in this research listed with Supplier companies

Appendix C: Enzymes and buffers used in this research listed with Supplier companies.

Appendix D: All sequencing analysis

Appendix E: All repeat IP experiment results

APPENDIX A

Table A: Chemicals and Commercial kits used in this study listed with Supplier companies and Cat No.

Chemicals and Commercial kits	Cat No	Supplier Companies
Acrylamide (40%, 29:1)	161-0146	BIO-RAD
Agarose	50004	SeaKem LE
Asetic Acid	6052	J.T.Baker
Coomassie Brilliant Blue	A3480	AppliChem
Dimethyl Sulfoxide	BP231	Fisher Scientific
Ethylenediamine Tetraacetic Acid	BP118	Fisher Scientific
Hydrochloric Acid (37%)	403872	Carlo Erba
Methanol	24229	Sigma Aldrich
Ponceau-S	BP103	Fisher Scientific
Pronasafe Nucleic Acid staining dye	CK130	Conda Lab
Sodium hydroxide pellets	480507	Carlo Erba
0.05% Trypsin-EDTA 1X	25300-054	Gibco
DMEM	319025-CL	Multicell
DMEM	BE12-614F	Lonza
DPBS w/o Ca, Mg	P04-36500	PAN
FBS	10270106	Gibco
L-Glutamine 200 mM 100X	25030-024	Gibco
PBS w/o Ca, Mg	BE17-516F	Lonza
Pen Strep	15140-122	Gibco
Agar	MC006	Labm
Ammonium Persulfate (APS)	BP179-100	Fisher Scientific
Bromophenol Blue	BP115	Fisher Scientific
BugBuster®Extraction Reagent	70584	Merck
D-Glucose Anhyrdous	0188	Amresco
di-sodium hydrogen phosphate	7558-79-4	Merck
Ethanol absolute	4146082	Carlo Erba
Glycerol	800689	MP
Glycine	808822	MP
Nonfat Dry Milk	9999	Cell Signaling
Nonidet®P40	A1694	AppliChem

Table A Cont: Chemicals and Commercial kits used in this study listed with Supplier companies and Cat No.

Chemical and commercial kits	Cat No.	Supplier Companies
Potassium dihydorgen phosphate	7778-77-0	Merck
SDS	194831	MP
Sodium chloride	7647-14-5	Merck
Sodium chloride	31434	Sigma-Aldrich
Tetramethyl-ethylenediamine	T7024	Sigma
Tris base	BP152-1	Fisher Scientific
Triton X-100®	BP151-100	Fisher Scientific
Trizma® base	T6066	Sigma
Tryptone	MC005	Labm
Tween® 20	C58H114O26	Fisher Scientific
Yeast extract	MC001	Labm
20X Lumiglo Reagent and 20X Peroxide	7003	Cell Signaling Tech.
GeneJet Plasmid Maxiprep	K0492	Thermo Scientific
GeneJet Plasmid Miniprep	K0503	Thermo Scientific
In-Fusion HD Cloning Kit	639648	Clontech
NucleoBond Xtra Maxi	740410	Macherey-Nagel
NucleoSpin Gel and PCR Clean-up	740609	Macherey-Nagel
NucleoSpin Plasmid DNA Purification Kit	740588	Macherey-Nagel
Pierce ECL Western Blotting Substrates	32106	Thermo Scientific
Pierce® BCA Protein Assay Kit	23227	Thermo Scientific
QuikChange Multi Site Directed Mutagenesis Kit	200515	Agilent
QuikChange Site Directed Mutagenesis Kit (XL)	200521	Agilent
Western Bright ECL	K-12045	Advansta
X-tremeGene HP Transfection Reagent	06366236001	Roche

APPENDIX B

Table B: Equipment used in this study are listed together with Supplier Companies.

Equipments	Supplier Companies
Agarose Gel Electrophoresis System	BIORAD, Sub-Cell GT Agarose Gel Electrophoresis System
Electronic pipettor	CAPP Controller
Gel Blot Paper	GB003
Hemocytometer	Marienfeld, Neubauer improved
Incubator	Binder, CB150
Microfuge Tubes	Axygen (1.5 ml, 0.6 ml, 0.2 ml) Nest (2 ml, 1.5 ml, 0.6 ml)
Micropipettes	Gilson 10 µl, 20 µl, 100 µl, 200 µl, 1 ml Socorex 10 µl, 200 µl, 1 ml
Microwave	Altus
Power Supply	BIORAD
SDS-PAGE System	BIORAD, Mini-Protean Tetra Cell
Serological pipettes	Sarstedt (25 ml, 10 ml, 5 ml, 2 ml) CAPP (25 ml, 10 ml, 5 ml)
Transfer System	BIORAD, Mini Trans-Blot Electrophoretic Transfer Cell
Tubes	Sarstedt (15 ml, 50 ml) Citotest (15 ml) Isolab (50 ml)
UltraCruz Nitrocellulose Membrane	Santa Cruz, sc3724
pH meter	Inolab wtw
Rotator	Stuart, SB3
Rotator Drive	Stuart, STR4
Shaker	Heidolph, Duomax 1030
Shaker	Sartorius, Certomat SII
Spectrophotometer	Shimadzu, UV-1700
Thermal Cycler	BIORAD T100
ThermoShaker	Biometra, TS1
Vortex	Heidolph, ReaxTop Harmony, LMS Group
Water Bath	ElektroMag, M96KP Mettler, WB110
Orbital shaker Hepa Model	Thermo, 481

Table B Cont: Equipment used in this study are listed together with Supplier Companies.

Equipments	Supplier companies
Autoclave	Zealway, GR
Centrifuge	ScanSpeed mini centrifuge, Labogene
	Beckman Coulter Allegra TM 25R
	VWR MicroStar 17R
	Beckman Coulter, A46473
	Thermo IEC CL10
Chemidoc	BIORAD, Chemidoc TM MP System
Freezer	Panasonic Ultra Low Temperature Freezer, MDF-U76V
Magnetic Stirrer	Wisd, MSH-20A
	VWR, VS-C77
Microplate reader	BIORAD, Benchmark Plus
Microscope	Olympus, M021
Nucleic acid spectrophotometer	NANODROP, ThermoFisher Scientific

APPENDIX C

Table C: Enzymes and buffers used in this study are listed together with Cat. No. and Supplier Companies.

Enzymes and Buffers	Cat No.	Supplier Companies
10X 2.11 Buffer	B7202	New England BioLabs
10X 3.1 Buffer	B7203	New England BioLabs
10X CutSmart Buffer	B7204	New England BioLabs
BamHI endonuclease	R3136	New England BioLabs
Bgl II endonuclease	ER0081	Thermo Scientific
BsrGI endonuclease	R0575	New England BioLabs
CloneAmp HiFi PCR Premix	638500	Clontech
MassRuler DNA Ladder Mix	SM0403	Thermo Scientific
MassRuler Low range DNA Ladder	SM0383	Thermo Scientific
NotI endonuclease	R0189S	New England BioLabs
NotI-HF endonuclease	R3189	New England BioLabs
Protein pre-stained marker	P7712	New England BioLabs
Protein pre-stained marker	P7709	New England BioLabs
Q5 ® High Fidelity DNA Polymerase	M0491	New England BioLabs
XbaI endonuclease	R0145S	New England BioLabs
XhoI endonuclease	R0146V	New England BioLabs
XmaI endonuclease	R0180S	New England BioLabs

APPENDIX D

Alignment of sequencing results after In-Fusion cloning reaction are listed below.

Sequencing result of FLAG-ATXN3 (sample name: **FA-4** sequenced with **T7** primer) aligned to pcDNA-FLAG-ATXN3 (Query).

Query	1	GCAGGCGTAGCAGCGTTTAACTTAGCTTGGTACCGAGCTCGGATCCACCATGGAGGACTA	60
Sbjct	1	GCAGGCGTAGCAGCGTTTAACTTAGCTTGGTACCGAGCTCGGATCCACCATGGAGGACTA	60
Query	61	CAAAGACGATGACGACAAGTGTAACAATGGAGTCCATCTTCCACGAGAAACAAGAAGGCTC	120
Sbjct	61	CAAAGACGATGACGACAAGTGTAACAATGGAGTCCATCTTCCACGAGAAACAAGAAGGCTC	120
Query	121	ACTTTGTGCTCAACATTGCCTGAATAACTTATTGCAAGGAGAATATTTTAGCCCCGTGGA	180
Sbjct	121	ACTTTGTGCTCAACATTGCCTGAATAACTTATTGCAAGGAGAATATTTTAGCCCCGTGGA	180
Query	181	ATTATCCTCAATTGCACATCAGCTGGATGAGGAGGAGAGGATGAGAATGGCAGAAGGAGG	240
Sbjct	181	ATTATCCTCAATTGCACATCAGCTGGATGAGGAGGAGAGGATGAGAATGGCAGAAGGAGG	240
Query	241	AGTTACTAGTGAAGATTATCGCACGTTTTACAGCAGCCTTCTGGAAATATGGATGACAG	300
Sbjct	241	AGTTACTAGTGAAGATTATCGCACGTTTTACAGCAGCCTTCTGGAAATATGGATGACAG	300
Query	301	TGGTTTTTCTCTATTAGGTTATAAGCAATGCCTTGAAAGTTTGGGGTTAGAACTAAT	360
Sbjct	301	TGGTTTTTCTCTATTAGGTTATAAGCAATGCCTTGAAAGTTTGGGGTTAGAACTAAT	360
Query	361	CCTGTTCAACAGTCCAGAGTATCAGAGGCTCAGGATCGATCCTATAAATGAAAGATCATT	420
Sbjct	361	CCTGTTCAACAGTCCAGAGTATCAGAGGCTCAGGATCGATCCTATAAATGAAAGATCATT	420
Query	421	TATATGCAATTATAAGGAACACTGGTTTACAGTTAGAAAATTAGGAAAACAGTGGTTTAA	480
Sbjct	421	TATATGCAATTATAAGGAACACTGGTTTACAGTTAGAAAATTAGGAAAACAGTGGTTTAA	480
Query	481	CTTGAATTCTCTTACGGGTCCAGAATTAATATCAGATACATATCTTGCACTTTTCTT	540
Sbjct	481	CTTGAATTCTCTTACGGGTCCAGAATTAATATCAGATACATATCTTGCACTTTTCTT	540
Query	541	GGCTCAATTACAACAGGAAGGTTATTCTATATTTGTCGTTAAGGGTGATCTGCCAGATTG	600
Sbjct	541	GGCTCAATTACAACAGGAAGGTTATTCTATATTTGTCGTTAAGGGTGATCTGCCAGATTG	600
Query	601	CGAAGCTGACCAACTCCTGCAGATGATTAGGGTCCAACAGATGCATCGACCAAACTTAT	660
Sbjct	601	CGAAGCTGACCAACTCCTGCAGATGATTAGGGTCCAACAGATGCATCGACCAAACTTAT	660
Query	661	TGGAGAAGAATTAGCACAACTAAAAGAGCAAAGAGTCCATAAAACAGACCTGGAACGAGT	720
Sbjct	661	TGGAGAAGAATTAGCACAACTAAAAGAGCAAAGAGTCCATAAAACAGACCTGGAACGAGT	720
Query	721	GTTAGAAGCAAATGATGGCTCAGGAATGTTAGACGAAGATGAGGAGGATTTGCAGAGGGC	780
Sbjct	721	GTTAGAAGCAAATGATGGCTCAGGAATGTTAGACGAAGATGAGGAGGATTTGCAGAGGGC	780
Query	781	TCTGGCACTAAGTCGCCAAGAAATTGACATGGAAGATGAGGAAGCAGATCTCCGAGGGC	840
Sbjct	781	TCTGGCACTAAGTCGCCAAGAAATTGACATGGAAGATGAGGAAGCAGATCTCCGAGGGC	840

Query	1381	CCTGGACCACAACCCCCCAGGCTGTACCTTCACAGTCCTGGTGCACACGAGAGAAGCCGC	1440
Sbjct	496	CCTGGACCACAACCCCCCAGGCTGTACCTTCACAGTCCTGGTGCACACGAGAGAAGCCGC	555
Query	1441	CACTCGCAACATGGAGAAGATCCAGGTCATCAAGGATTTCCCCTGGATCCTGGCGGATGA	1500
Sbjct	556	CACTCGCAACATGGAGAAGATCCAGGTCATCAAGGATTTCCCCTGGATCCTGGCGGATGA	615
Query	1501	GCAGGATGTCCACATGCATGACCCCCGGCTGATACCACTAAAAACCATGACGTCGGACAT	1560
Sbjct	616	GCAGGATGTCCACATGCATGACCCCCGGCTGATACCACTAAAAACCATGACGTCGGACAT	675
Query	1561	TTTAAAGATGCAGCTTTGCGTGGAGAGCGCGCTCATAAAGGCAGCTGATCTAGAGGGCC	1620
Sbjct	676	TTTAAAGATGCAGCTTTGCGTGGAGAGCGCGCTCATAAAGGCAGCTGATCTAGAGGGCC	735
Query	1621	CGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTG	1680
Sbjct	736	CGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTG	795
Query	1681	CCCCCCCCGTCCTTCTTGACCTGGAGGTGCCACTCCCCTGTCCTTTCTAATA	1740
Sbjct	796	CCCCCCCCGTCCTTCTTGACCTGGAGGTGCCACTCCCCTGTCCTTTCTAATA	855
Query	1741	AAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTATTCTATTCTgggggggtgggggt	1800
Sbjct	856	AAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTATTCTATTCTGGGGGGTGGGGT	915
Query	1801	ggggCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGT	1860
Sbjct	916	GGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCGGGCATGCGGGGGATGCGGC	975
Query	1861	GGGCTCTATGGCTTCTGAGGCGGAAAGAACCAAGCTGGGGCTCTAGGGGGTATCCCCACG-	1919
Sbjct	976	GGGGTCTATGGTTTCTGAGGGGGAAAGAACCAACTGGGGCTCTAGGGGGTATCCC-ACGC	1034
Query	1920	CGCCCTGTAGCGGCGCATTAAG	1941
Sbjct	1035	CGCCCGGGAGCTGCACACTAAG	1056

Sequencing result of HA-ATXN3 (sample name: **HA-AT3-1** sequenced with **CMV Forward** primer) aligned to pcDNA-HA-ATXN3 (Query).

Query	810	AGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACG	869
Sbjct	16	AGCAGAGCTCTCTGGCTAACTAGAGAACCCACTGCTTACTGGCTTATCGAAATTAATACG	75
Query	870	ACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAACTTAAGCTTGGTACCGAGCTCG	929
Sbjct	76	ACTCACTATAGGGAGACCCAAGCTGGCTAGCGTTTAACTTAAGCTTGGTACCGAGCTCG	135
Query	930	GATCACCACCATGGAGTACCCATACGATGTTCCAGATTACGCTCGAGAGATGGAGTCCAT	989
Sbjct	136	GATCACCACCATGGAGTACCCATACGATGTTCCAGATTACGCTCGAGAGATGGAGTCCAT	195
Query	990	CTTCCACGAGAAACAAGAAGGCTCACTTTGTGCTCAACATTGCCTGAATAACTTATTGCA	1049
Sbjct	196	CTTCCACGAGAAACAAGAAGGCTCACTTTGTGCTCAACATTGCCTGAATAACTTATTGCA	255
Query	1050	AGGAGAATATTTTAGCCCCGTGGAATTATCCTCAATTGCACATCAGCTGGATGAGGAGGA	1109
Sbjct	256	AGGAGAATATTTTAGCCCCGTGGAATTATCCTCAATTGCACATCAGCTGGATGAGGAGGA	315
Query	1110	GAGGATGAGAATGGCAGAAGGAGGAGTTACTAGTGAAGATTATCGCACGTTTTTACAGCA	1169
Sbjct	316	GAGGATGAGAATGGCAGAAGGAGGAGTTACTAGTGAAGATTATCGCACGTTTTTACAGCA	375
Query	1170	GCCTTCTGGAAATATGGATGACAGTGGTTTTTCTCTATTAGGTTATAAGCAATGCCTT	1229
Sbjct	376	GCCTTCTGGAAATATGGATGACAGTGGTTTTTCTCTATTAGGTTATAAGCAATGCCTT	435
Query	1230	GAAAGTTTGGGGTTTAGAACTAATCCTGTTCAACAGTCCAGAGTATCAGAGGCTCAGGAT	1289
Sbjct	436	GAAAGTTTGGGGTTTAGAACTAATCCTGTTCAACAGTCCAGAGTATCAGAGGCTCAGGAT	495
Query	1290	CGATCCTATAAATGAAAGATCATTTATATGCAATTATAAGGAACACTGGTTTACAGTTAG	1349
Sbjct	496	CGATCCTATAAATGAAAGATCATTTATATGCAATTATAAGGAACACTGGTTTACAGTTAG	555

Sequencing result of GST-ATXN3 (sample name: **GST-AT3-4** sequenced with **PGEX 5'** primer) aligned to PGEX-4T3-ATXN3 (Query).

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Query  911  ATCGGATCTGGTTCCGCGTGGATCCATGGAGTCCATCTTCCACGAGAAACAAGAAGGCTC  970
Sbjct   12  ATCGGATCTGGTTCCGCGTGGATCCATGGAGTCCATCTTCCACGAGAAACAAGAAGGCTC  71

Query  971  ACTTTGTGCTCAACATTGCCTGAATAACTTATTGCAAGGAGAATATTTAGCCCCGTGGA  1030
Sbjct   72  ACTTTGTGCTCAACATTGCCTGAATAACTTATTGCAAGGAGAATATTTAGCCCCGTGGA  131

Query 1031  ATTATCCTCAATTGCACATCAGCTGGATGAGGAGGAGAGGATGAGAATGGCAGAAGGAGG  1090
Sbjct  132  ATTATCCTCAATTGCACATCAGCTGGATGAGGAGGAGAGGATGAGAATGGCAGAAGGAGG  191

Query 1091  AGTTACTAGTGAAGATTATCGCACGTTTTTACAGCAGCCTTCTGGAAATATGGATGACAG  1150
Sbjct  192  AGTTACTAGTGAAGATTATCGCACGTTTTTACAGCAGCCTTCTGGAAATATGGATGACAG  251

Query 1151  TGGTTTTTCTCTATTCAAGTTATAAGCAATGCCTTGAAAGTTTGGGGTTTGAAGTAAT  1210
Sbjct  252  TGGTTTTTCTCTATTCAAGTTATAAGCAATGCCTTGAAAGTTTGGGGTTTGAAGTAAT  311

Query 1211  CCTGTTCAACAGTCCAGAGTATCAGAGGCTCAGGATCGATCCTATAAATGAAAGATCATT  1270
Sbjct  312  CCTGTTCAACAGTCCAGAGTATCAGAGGCTCGGGATCGATCCTATAAATGAAAGATCATT  371

Query 1271  TATATGCAATTATAAGGAACACTGGTTTACAGTTAGAAAATTAGGAAAACAGTGGTTTAA  1330
Sbjct  372  TATATGCAATTATAAGGAACACTGGTTTACAGTTAGAAAATTAGGAAAACAGTGGTTTAA  431

Query 1331  CTTGAATTCTCTCTTGACGGGTCCAGAATTAATATCAGATACATATCTTGCACTTTTCTT  1390
Sbjct  432  CTTGAATTCTCTCTTGACGGGTCCAGAATTAATATCAGATACATATCTTGCACTTTTCTT  491

Query 1391  GGCTCAATTACAACAGGAAGGTTATTCTATATTTGTCGTTAAGGGTGATCTGCCAGATTG  1450
Sbjct  492  GGCTCAATTACAACAGGAAGGTTATTCTATATTTGTCGTTAAGGGTGATCTGCCAGATTG  551

Query 1451  CGAAGCTGACCAACTCCTGCAGATGATTAGGGTCCAACAGATGCATCGACCAAAACTTAT  1510
Sbjct  552  CGAAGCTGACCAACTCCTGCAGATGATTAGGGTCCAACAGATGCATCGACCAAAACTTAT  611

Query 1511  TGGAGAAGAATTAGCACAACATAAAGAGCAAAGAGTCCATAAAACAGACCTGGAACGAGT  1570
Sbjct  612  TGGAGAAGAATTAGCACAACATAAAGAGCAAAGAGTCCATAAAACAGACCTGGAACGAGT  671

Query 1571  GTTAGAAGCAAATGATGGCTCAGGAATGTTAGACGAAGATGAGGAGGATTTGCAGAGGGC  1630
Sbjct  672  GTTAGAAGCAAATGATGGCTCAGGAATGTTAGACGAAGATGAGGAGGATTTGCAGAGGGC  731

Query 1631  TCTGGCACTAAGTCGCCAAGAAATTGACATGGAAGATGAGGAAGCAGATCTCCGCAGGGC  1690
Sbjct  732  TCT-GCACTAAGTCGCCAAGAAATTGACATGGAAGATGAGGAAGCAGATCTCCGCAGGGC  790

Query 1691  TATTCAAGTAAGTATGCAAGGTAGTTCCAGAAACATATCTCAAGATATGACACAGACATC  1750
Sbjct  791  TATTCAAGTAAGTATGCAAGGTAGTTCCAGAAACATATCTCAAGATATGACACAGACATC  850

Query 1751  AGGTACAAATCTTACTTCAGAAGAGCTTCGGAAGAGACGAGAAGCCTACTTTGaaaaaca  1810
Sbjct  851  AGGTACAAATCTTACTTCAGAAGAGCTTCGGAAGAGACGAGAAGCCTACTTTGAAAAACA  910

Query 1811  gcagcaaaagcagcaacagcagcagcagcagcagcagcagcagcagcagcagcagcagca  1870
Sbjct  911  GCAGCAAAAGCAGCAACAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCA  970

Query 1871  gcagcagGGGGACCTATCAGGACAGAGTTCACATCCATGTGAAAGGCCAGCCACCAAGTTC  1930
Sbjct  971  GCAGCAGGGGGACCTATCAGGACAGAGTTCACATCCATGTGAAAGGCCAGCCACCAAGTTC  1030

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Query	1931	AGGAGCACTTGGGAGTGATCTAGGTGATGCTATGAGTGAAGAAGACATGCTTCAGGCAGC	1990
Sbjct	1031	AGGAGCACTTGGGAGTGATCTAGGTGATGCTATGAGTGAAGAAGACATGCTTCAGGCAGC	1090
Query	1991	TGTGACCATGTCTTTAGAAACTGTCAGAAATGATTTGAAAACAGAAAGGaaaaaaTAATC	2050
Sbjct	1091	TGTGACCATGTCTTTAGAAACTGTCAGAAATGATTTGAAAACAGAAAGGAAAAAATAATC	1150
Query	2051	GAGCGGCCGCATCGTGACTGACTGACGATCTGCCTCGCG-CGTTTCGGTGATGACGGTGA	2109
Sbjct	1151	GAGCGGCCGCATCTTGAC-GACTGACGATTTGCCTC-CGCCGTTTCGGGGATGACGGTGA	1208
Query	2110	AAACCTCTGACACA-TGCAGCTCCCGGAGA-CGGTCACAGC-TTGTCTGTAAGCGGA-TG	2165
Sbjct	1209	AAACCTCTGGAACATTGCAACTCCCCGAAAACGGTTCCAACTTTGTCTGTAAACGGATTG	1268
Query	2166	CCGGGAGCAGACA-AGCCCGTCAGGGCGCGTC-AGCGGGTGTT--GGCGGGTGTCGGGGC	2221
Sbjct	1269	CCGGGAACAAACAGAGCCCTTCAGGGCCCGCCACCGGGGGTTTTGGCGGGTGTCGGGGG	1328
Query	2222	GC-AGCC-ATGA--CCAGTCACGTAGCG-ATA-GCGG-AGTGTAT-AATT-CTTGAAG-	2271
Sbjct	1329	GCCACCCCATGACCCCATTCCTTACCAGAAAAGCGGGAGTGGATTAATTTCTTGAAAC	1388
Query	2272	ACGAAAGGGCCTC	2284
Sbjct	1389	ACAAAAGGGCCTC	1401



APPENDIX E

All Co-immunoprecipitation and GST pulldown experiment results are listed below:

Figure E.1: Repeat of Figure 3.6.

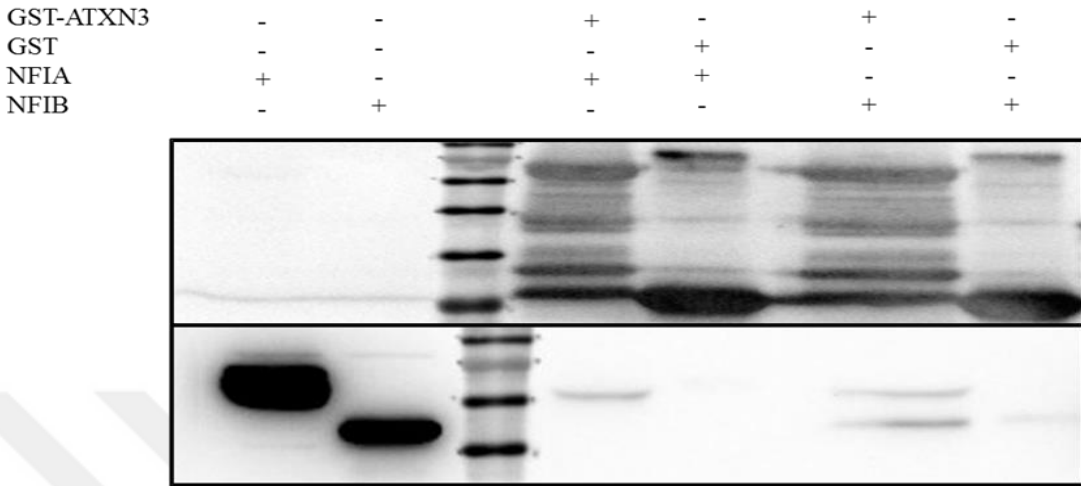


Figure E.2: Repeat of Figure 3.7.

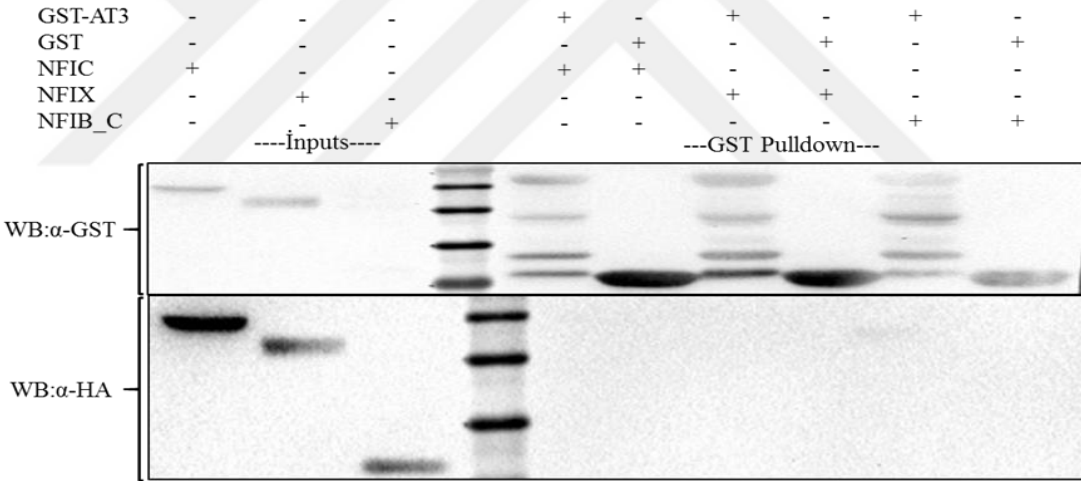


Figure E.3: Repeat of Figure 3.9.

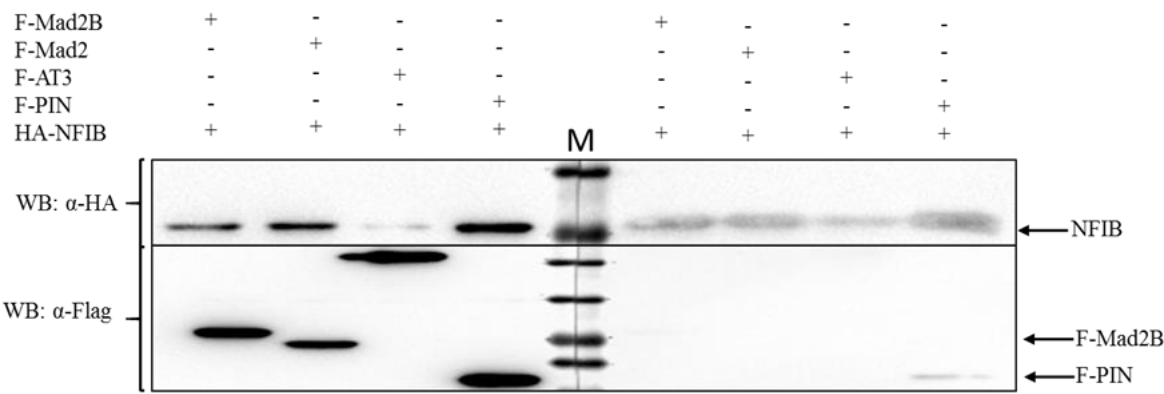


Figure E.4: Repeat of Figure 3.10.

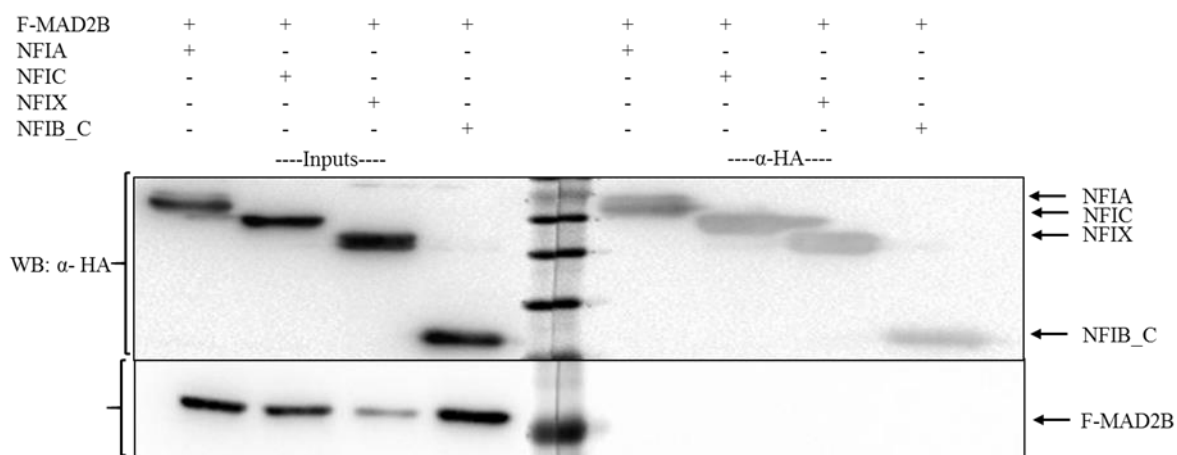


Figure E.5: Repeat of Figure 3.11.

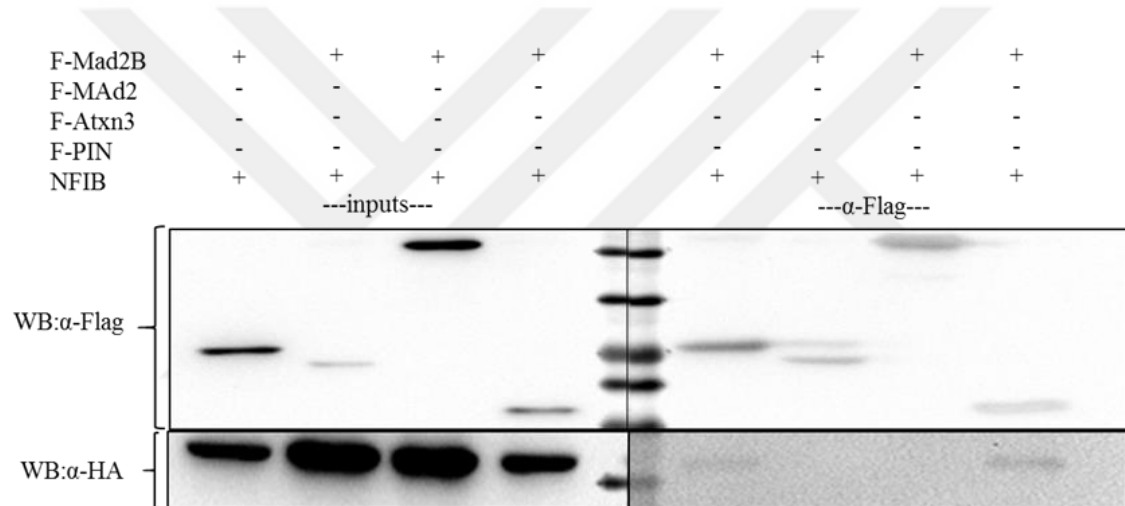
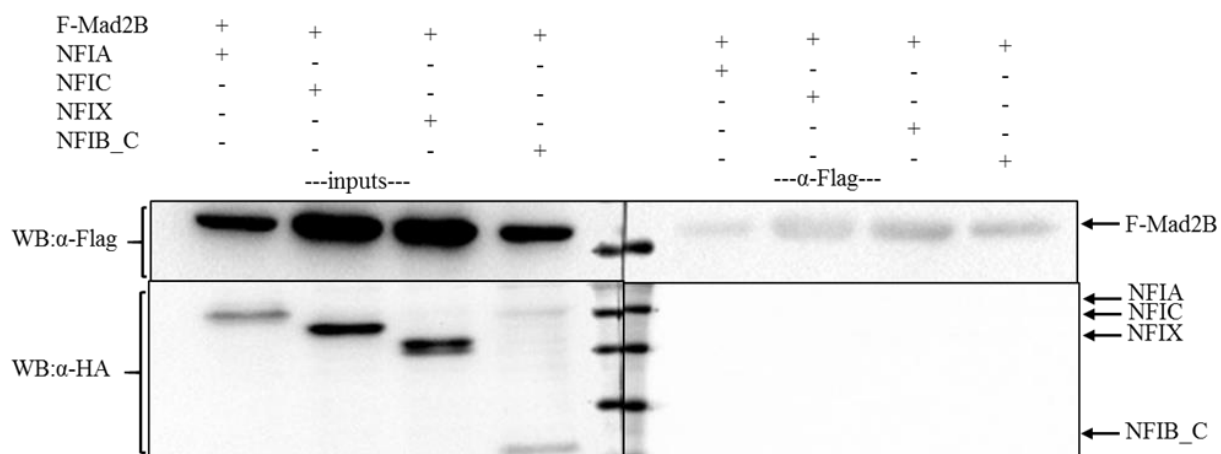


Figure E.6: Repeat of Figure 3.12.



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