ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

INVESTIGATION OF THE INTERACTION BETWEEN NFI AND ITS POTENTIAL INTERACTION PARTNER BRG1

M.Sc. THESIS

Selim TÜRKEL

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Thesis Advisor: Assist. Prof. Dr. Aslı KUMBASAR

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

NFI PROTEİNİNİN POTANSİYEL ETKİLEŞİM PARTNERİ OLAN BRG1 İLE ETKİLEŞİMİNİN ARAŞTIRILMASI

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MART 2018

Selim TÜRKEL, a M.Sc. student of ITU Graduate School of Science, Engineering and Technology, Student ID 521141117, successfully defended his thesis/dissertation entitled "INVESTIGATION OF THE INTERACTION BETWEEN NFI AND ITS POTENTIAL INTERACTION PARTNER BRG1", which he prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 17 November 2017
Date of Defense : 8 March 2018

To my family and friends,

FOREWORD

First of all, I would like to express my gratitude to my thesis advisor Assist. Prof. Aslı KUMBASAR for her support and guidance throughout my thesis project and graduate study, and for helping me become a better scientist.

I would like to thank my thesis committee so much for sparing their time for me and helping me to improve my thesis.

I owe appreciation to Istanbul Technical University's Department of Scientific Research Projects (BAP) for the financial support with the project number 40249.

I owe many thanks to my former and current Kumbasar Lab mates: Varol GÜLER, Betül KUTAY, Sinem SARITAŞ and Dicle PINAR for their friendship and help, Eda YILMAZYILDIRIM and Cemre LEKTEMUR for teaching me each laboratory technique patiently and trying to help me with all my questions, Ece ÇAĞDAŞ and İbrahim OĞUZ for their warm friendship and support inside and outside the laboratory.

In addition, I want to thank all MOBGAM members for their friendship and for providing a good research environment.

Many special thanks to Rana FUÇUCUOĞLU for her companionship inside and outside the laboratory, for her assistance and support in every respect, and for making my life better throughout my graduate study.

Last but not least, I would like to thank my parents Hanım TÜRKEL and Mehmet TÜRKEL, my brother Enes TÜRKEL and my sister Nurgül TÜRKEL and my cousin Cem KARA for always being there and for their precious support and affection.

March 2018

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Molecular Biologist

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ABBREVIATIONS

α-	: Anti-
µg	**:** Microgram
µl	**:** Microliter
µm	**:** Micrometer
µM	**:** Micromolar
^{o}C	**:** Celsius
A	**:** Adenine
APS	**:** Ammonium persulfate
ARID1a/b	**:** AT-rich interactive domain-containing protein 1a/b
ASD	**:** Autism spectrum disorder
ATP	**:** Adenosine triphosphate
ATPase	**:** Adenosine triphosphatase
ATXN3	**:** Ataxin-3
BAF	**:** BRG1/BRM Associated Factor
BAF170	**:** BRG1-Associated Factor 170
BAP	**:** Brahma-associated protein
BCA	**:** Bicinchoninic acid
Brm	**:** Brahma
BSA	**:** Bovine serum albumin
bp	**:** Base pair
Brg1	**:** Brahma related gene I
C	**:** Cytosine
C. elegans	**:** Caenorhabditis elegans
CGN	**:** Cerebellar granule neuron
Co-IP	**:** Co-immunoprecipitation
CO_2	**:** Carbon dioxide
CTIP2	**:** Chicken ovalbumin upstream promoter transcription factor interacting protein 2
CTF	**:** CAAT box transcription factor
Cu	**:** Copper
CUX1	**:** Cut like homeobox 1
dH_2O	**:** Distilled water
DMEM	**:** Dulbecco's Modified Eagle Medium
DMSO	**:** Dimethyl sulfoxide
DNA	**:** Deoxyribonucleic acid
DPBS	**:** Dulbecco's Phosphate-Buffered Saline
DTT	**:** Dithiothreitol
ECL	**:** Enhanced chemiluminescence
E.coli	: *Escherichia coli*
EDTA	**:** Ethylendiaminetetraacetic acid
EGTA	**:** Egzatic acid
Etv1	**:** ETS Variant 1

EZH2 : Enhancer of zeste homolog 2
FBS : Fetal Bovine Serum
F-Baf170 : FLAG-tagged Baf170
F-Brg1 : FLAG-tagged Brg1
F-Mad2 : FLAG-tagged Mad2
g : Gram
G : Guanine
GLI2 : GLI Family Zinc Finger 2
H_2O : Water
HA : Hemagglutinin
HA-NFI : HA-tagged NFI
HA-ATXN3 : HA-tagged ATXN3
HCl : Hydrochloric acid
HDAC1 : Histone deacetylase 1
HEK293T : Human embryonic kidney 293T
HELICc : Helicase superfamily C-terminal domain
HSA : Helicase-SANT-associated domain
IPC : Intermediate progenitor cell
kb : Kilobase
kDa : Kilodalton
L : Liter
LB : Luria-Bertani
M : Molar
Mad2 : Mitotic arrest deficient 2
mBAF : Mammalian BAF complex
Mef2c : Myocyte Enhancer Factor 2C
mg : Miligram
min : Minute
MITF : Microphthalmia-associated transcription factor
ml : Mililiter
mm : Milimeter
mM : Milimolar
mol : Moles
mRNA : Messenger RNA
NaCl : Sodium chloride
NaOH : Sodium hydroxide
nBAF : Neuronal BAF complex
npBAF : Neural progenitor BAF complex
NFATc4 : Nuclear factor of activated T-cells, cytoplasmic 4
NFI : Nuclear Factor I
ng : Nanogram
NLS : Nuclear localization signal
nm : Nanometer
NRC : Nucleosome remodeling complexes
NSC : Neural stem cell
NP-40 : Nonidet P40
OCT4 : Octamer-binding transcription factor 4
olBAF : Oligodendrocyte BAF complex
PAX6 : Paired box 6
PCR : Polymerase Chain Reaction

pH	**:** Potential hydrogen
PBS	**:** Phosphate-Buffered Saline
PEI	**:** Polyethylenimine
PHD	**:** Plant homeodomain
pmol	**:** Picomolar
QLQ	**:** Glutamine-Leucine-Glutamine (Gln-Leu-Gln) motif
REST	**:** Repressor Element 1-Silencing Transcription factor
RNase	**:** Ribonuclease
rpm	**:** Revolutions per minute
SANT	**:** Switching-defective protein 3 (Swi3), Adaptor 2 (Ada2), Nuclear receptor corepressor, Transcription factor IIIB
scBAF	: Schwann cell BAF complex
SDS	**:** Sodium Dodecyl Sulfate
SDS-PAGE	**:** Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
sec	**:** Second
Smad3	**:** SMAD Family Member 3
Smarca4	**:** SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4
SMARCAL1	**:** SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A Like 1
Sox10	**:** SRY-Box 10
STAT5	**:** Signal Transducer And Activator Of Transcription 5A
SVZ	**:** Subventricular zone
SWI/SNF	**:** SWItch/Sucrose Non-Fermentable
T	**:** Thymine
TAE	**:** Tris-acetate-EDTA
Tag-1	**:** Transient axonal glycoprotein-1
TBS	**:** Tris-buffered saline
TBS-T	**:** Tris-buffered saline with Tween
TGFβ	**:** Transforming Growth Factor Beta
TF	**:** Transcription factor
Temed	**:** N,N,N',N'-Tetramethyl ethylenediamine
Tris	**:** Hydroxymethyl aminomethane
UV	**:** Ultraviolet
V	**:** Volt
w/v	**:** Weight / volume
x g	**:** times gravity

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INVESTIGATION OF THE INTERACTION BETWEEN NFI AND ITS POTENTIAL INTERACTION PARTNER BRG1

SUMMARY

Nuclear factor I (NFI) protein family of transcription factors regulate gene expression and viral DNA replication via binding to certain DNA sequences. There are four different NFI genes (NFIA, NFIB, NFIC and NFIX) of which sequences are conserved between vertebrates. The four vertebrate NFI genes are alternatively spliced to bring about multiple transcript variants from each gene. NFI proteins bind to TTGGC(N5)GCCAA sequences on double-stranded DNA with high affinity, and to TTGGC and GCCAA sequences with low affinity. NFI protein family share an N-terminal region for DNA-binding and dimerization, and a C-terminal region that activate/repress gene expression. The N-terminal site is a 220 amino acid long structure that is conserved between all vertebrates. This site includes four cysteine amino acids. Three of the cysteines participate in DNA binding and dimerization. The C-terminal site of NFI is the region that varies between the four NFI genes. Alternative splicing of the C-terminal site generates various NFI proteins from each gene. This site functions in the repression or activation of the expression from target genes. Therefore, NFI proteins are also known as transcription factors.

Three NFI family members, NFIA, NFIB and NFIX play important roles in neural development. Defects in the development of cerebral cortex, hippocampus as well as, spinal cord and cerebellum in *Nfi* knockouts mice show that NFIs are required for central nervous system development. Expression of all three nervous system specific NFIs are detected in neurons, astrocytes and oligodendrocytes.

NFI transcription factors interact with a variety of genes and proteins to regulate gene expression. In the hindbrain NFIA interacts with other transcription factors, such as Sox9 and Sox10 to regulate glial differentiation. NFIs can also bind chromatin modifiers: co-immunoprecipitation and co-localization experiments in HeLa cells and T lymphocytes carried out by Zhao L. et al (2005) show that the NFI transcription factor binds to BRG1 and the RNA polymerase II enzyme in order to activate transcription.

BRG1/BRM Associated Factor (BAF) complex is an epigenetic factor that uses its Brahma Associated Gene 1 (BRG1) ATPase subunit to utilize the released ATP energy and in this way alters the target chromatin structures. Although similar in structure to the yeast SWI/SNF complex, the mammalian BAF complex is 2 megadaltons size and has at least 15 distinct subunits. Combinations of various subunits present in different cell types can generate numerous distinct BAF complexes engaged in differential interactions.

The BAF complex can interact with transcription factors in order to activate or repress expression of a target gene. Therefore, its function is essential for many

developmental processes. For instance, *Brg1*$^{-/-}$ mouse exhibits neural tube formation defects in the early stages of mouse development. Silencing of *Brg1* expression in neural progenitor cells prevents regeneration of and thereby causes a decrease in the number of neural cells.

As BRG1 and NFI both are involved in regulation of gene expression during development and had been shown to interact in nonneural cells, we wanted to explore this interaction further. We carried out overexpression and co-immunoprecipitation studies to determine if these interactions are found in neural cell lines and are NFI member specific. To this end, epitope tagged BRG1 and NFI proteins were overexpressed in HEK293T cells and their interaction was analyzed by co-immunoprecipitation experiments. We showed that FLAG-BRG1 is precipitated by all HA-NFI family members. We also showed by reciprocal co-immunoprecipitation experiments NFIB and NFIX are indeed precipitated by BRG1, while preliminary data indicate that NFIA and NFIC may also interact with BRG1. In addition, we performed co-immunoprecipitation experiments using SH-SY5Y neuroblastoma nuclear extracts enriched in endogenous BRG1 and NFI proteins. Interestingly, this cell line expresses NFIB in higher amounts when compared to other NFI proteins. We were unable to determine if NFIB is precipitated by BRG1, studies using different primary antibodies and antibody controls are undergoing to obtain definitive results.

BRG1 protein comprises four different domains. The first domain is a Proline-rich domain that plays a role in protein-protein interactions. The second domain is a conserved region of unknown function. The third domain is the enzymatic ATPase domain that is involved in chromatin remodeling whereas the fourth domain is involved in the interaction of BRG1 with acetylated histone tails. In this study, we also wanted to identify the domains through which BRG1 interacts with NFIB. We set out to generate mammalian expression constructs including Myc-tagged BRG1 domains I and II by recombination and obtained vectors encoding Myc-tagged BRG1 domains III and IV and the full-length BRG1 from Joan Massague. Preliminary experiments employing anti-HA antibodies and the domains III and IV showed that these epitope tagged proteins may interact nonspecifically with anti-HA resin. Co-immunoprecipitation experiments where BRG1 domains will be carried out to determine whether NFI proteins do precipitate with any of these domains.

NFI PROTEİNİNİN POTANSİYEL ETKİLEŞİM PARTNERİ OLAN BRG1 İLE ETKİLEŞİMİNİN ARAŞTIRILMASI

ÖZET

Nükleer faktör I (NFI) protein ailesi transkripsiyon faktörleri belirli DNA bölgelerine bağlanarak hem viral DNA replikasyonunu hem de gen ifadesini kontrol eder. NFIA, NFIB, NFIC ve NFIX olmak üzere dört adet NFI geni bulunmaktadır ve bu genler omurgalı canlılar arasında korunmuş yapıdadır. Omurgalılarda bulunan dört adet NFI geninin gen ifadesi sırasında alternatif eşlenmesi sonucunda birçok NFI protein çeşidi üretilebilmektedir. NFI proteinleri çift zincirli DNA üzerindeki TTGGC(N5)GCCAA sekansına yüksek afinite ile, TTGGC ve GCCAA sekansına ise düşük afinite ile bağlanmaktadır. NFI protein ailesi N-ucunda DNA'ya bağlanma ve dimerizasyon bölgesini, C-ucunda ise gen ifadesini baskılama veya aktivasyon bölgelerinden oluşmaktadır. N-ucu bölgesi tüm omurgalı NFI ailesinde yaklaşık 220 amino asit içeren çok korunaklı bir yapı halindedir. Bu bölge dört adet sistein amino asidi içermektedir. Sistein amino asitlerinin üç tanesi NFI proteininin DNA'ya bağlanmasında ve dimerizasyonda rol oynamaktadır. Dört adet NFI geninin farklı olmasını sağlayan bölge NFI'in C-ucudur. C-ucunun alternatif eşlenme geçirmesi sonucunda her genden farklı NFI proteinleri üretilmektedir. Bu bölge NFI proteininin hedef gen bölgelerinin ifadesini baskılamasında veya aktifleştirmesinde görevlidir. Bu nedenle NFI, transkripsiyon faktörü olarak da bilinmektedir.

NFI transkripsiyon faktörü ailesinin üç üyesi, NFIA, NFIB ve NFIX nöral gelişimde önemli roller oynamaktadır. *Nfi* nakavt deneylerinde hipokampüs, omurilik, pons gelişimlerinde bozukluklar meydana gelmesi NFI'in nöral gelişimde rol oynadığının göstergesidir. Sinir sistemine spesifik olan bu üç NFI proteininin ifadesi nöronlarda, astrositlerde ve oligodendrositlerde tespit edilmiştir.

NFI transkripsiyon faktörleri çeşitli genler ve proteinler ile etkileşerek gen ifadesini düzenlemektedir. Arka beyinde NFIA, Sox9 ve Sox10 gibi diğer transkripsiyon faktörleri ile etkileşerek gliyal farklılaşmayı düzenler. NFI proteinleri ayrıca diğer kromatin düzenleyiciler ile de etkileşir: Zhao L. ve arkadaşlarının (2005) HeLa ve T lenfosit hücrelerinde yaptığı birlikte çöktürme ve kolokalizasyon deneyleri, NFI transkripsiyon faktörünün transkripsiyonu aktifleştirmek üzere BRG1'e ve RNA polimeraz II enzimine bağlandığını göstermektedir.

BRG1/BRM İlişkili Faktör (BAF) kompleksi, yapısında bulunan BRG1 (Brahma İlişkili Gen 1) ATPase alt ünitesi sayesinde ATP'yi enerji kaynağı olarak kullanan ve bu sayede kromatinin yapısını değiştiren bir epigenetik faktördür. Maya SWI/SNF kompleksine benzeyen bir yapıda bulunan bu memeli kompleksi, 2 megadalton büyüklüğündedir ve en az 15 alt üniteye sahiptir. Farklı hücrelerde bulunan çeşitli alt ünite kombinasyonları, birçok farklı etkileşimde bulunabilecek sayısız BAF kompleksleri meydana getirebilir.

BAF kompleksi transkripsiyon faktörleri ile etkileşime girerek hedef genleri transkripsiyona aktif veya pasif hale getirebilmektedir. Bu özelliğinden dolayı birçok gelişimsel proseslerde önemli roller oynamaktadır. Örneğin *Brg1*$^{-/-}$ farelerde embriyo gelişiminin erken safhalarında nöral tüp gelişiminde kusurlar oluşmaktadır. *Brg1* ifadesinin nöral öncül hücrelerde susturulması, nöral hücrelerin kendilerini yenilemelerini engellemekte ve bu nedenle nöral hücre miktarında azalma görülmektedir.

BRG1 (Brahma İlişkili Gen 1) BAF kompleksinin enzimatik alt ünitesidir. ATP'yi enerji kaynağı olarak kullanarak kromatinin yapısını değiştirir ve bu sayede hedef genin gen ifadesine açık veya kapalı hale gelmesini sağlar. Yapısında bir çok bölge bulundurur. N-ucundaki QLQ bölgesi ile proteinler ile etkileşime girer, ayrıca yine N-ucundaki HSA bölgesi ile aktin ve aktin ilişkili proteinler ile etkileşime girmektedir. N-ucunda bulunan bir başka bölge olan BRK bölgesinin görevi henüz bilinmemektedir. BRG1 proteininin orta kısmında DEXHc ve HELICc enzimatik bölgeleri bulunmaktadır. Bu bölgeler helikaz aktivitesi gösterir ve enzimatik işlemler için ATP'yi enerji kaynağı olarak kullanmaktadırlar. BRG1'nın C-ucunda ise Bromo bölgesi ve AT-hook motifi bulunmaktadır. Bromo bölgesi, BRG1'nın histon kuyrukları üzerinde bulunan asetillenmiş lizin rezidülerine bağlanmasını sağlar. AT-hook motifi ise BRG1'nın DNA'ya bağlanmasına ve asetillenmiş histon bölgelerine getirilmesine yardımcı olduğu düşünülmektedir.

Hem BRG1 hem de NFI gelişim sırasında gen ifadesinin regülasyonunda yer aldığı ve nöral olmayan hücrelerde etkileştikleri gösterildiği için bu etkileşimi daha fazla araştırmak istedik. Bu etkileşimlerin nöral hücre hatlarında bulunup bulunmadığını ve NFI üyelerine spesifik olup olmadığını belirlemek için ifade arttırımı ve birlikte çöktürme deneyleri gerçekleştirdik. Bu amaçla, epitop işaretli BRG1 ve NFI proteinlerinin ifadesi HEK293T hücrelerinde arttırıldı ve etkileşimleri birlikte çöktürme deneyleri ile analiz edildi. FLAG-BRG1'in bütün HA-NFI ailesi proteinleri ile çöktüğünü gösterdik. Karşılıklı birlikte çöktürme deneyleri ile Nfib ve Nfix'in BRG1 ile çöktüğünü gösterdik. Öncül verilere göre de Nfia ve Nfic'nin BRG1 ile etkileşebileceğini düşünmekteyiz.

Ek olarak, endojen NFIB ve BRG1 proteinleri zenginleştirilmiş SH-SY5Y nöroblastoma nükleer ekstraklarını kullanarak immünçöktürme deneyleri gerçekleştirdik. İlginçtir ki, NFIB bu hücre hattında diğer NFI ailesi üyelerine göre daha fazla miktarda ifade edilmektedir. Ancak endojen seviyede eksprese olan BRG1'in NFIB'yi çöktürdüğünü doğrulayamadık, kesin sonuçlar elde edebilmek için farklı primer antikorlar ve antikor kontrolleri ile çalışmalar devam etmektedir.

BRG1 proteini dört farklı bölgeden oluşmaktadır. İlk bölge Prolin yönünden zengin protein-protein etkileşiminde rol oynayan, ikinci bölge fonksiyonu bilinmeyen korunaklı bir yapıya sahip, üçüncü bölge enzimatik reaksiyonlarda rol oynayan, son bölge ise BRG1'in asetillenmiş histon kuyruğuna bağlanmasını sağlayan bölgelerdir. Bu çalışmada, BRG1'in hangi bölgeleriyle NFI ile etkileştiğini de tespit etmek istedik. Rekombinasyon ile Myc işaretli BRG1 bölgeleri I ve II'yi içeren memeli ifade konstraktları üretmeye çalıştık ve Joan Massague'un laboratuvarından Myc işaretli BRG1 bölgeleri III ve IV'ü ve FLAG işaretli tüm BRG1'i içeren vektörler edindik. Ancak In-fusion klonlama ile BRG1'nın I ve II bölgelerini klonlamakta başarılı olamadık. Bu nedenle birlikte çöktürme deneylerimize elimizde bulunan III ve IV bölgeleri ile devam ettik. III ve IV bölgeleri ile HA işaretli Nfib arasındaki etkileşimi bulmak için anti-HA rezini kullanılarak yapılan birlikte çöktürme

deneylerinde Myc işaretli BRG1 III ve IV bölgelerinin Nfib'nin yanında negatif kontrol ile de çöktüğünü belirledik. Yapılan bu öncül deneylerde epitop işaretli bu proteinlerin anti-HA resin ile nonspesifik olarak etkileşiyor olabileceği sonucuna ulaştık. Bu BRG1 bölgelerinin NFI proteinleri ile beraber çöküp çökmeyeceği birlikte çöktürme deneyleri ile incelenecektir.

1. INTRODUCTION

1.1 Nuclear Factor I (NFI/CTF) Gene Family

The Nuclear Factor I (NFI) is a transcription factor family whose members are also known as CTF or CAAT box transcription factors, functioning in regulation of gene expression. NFIs are first reported to play a role in replication of viral DNA. NFI protein was initially discovered in nuclear extracts of HeLa cells as the activity that promotes initiation of adenoviral DNA replication (Nagata et al., 1982). Prokaryotes do not possess any NFI gene but the nematode worm *C. elegans* has a single Nfi (Fletcher et al., 1999). The NFI family is composed of four members in vertebrates: NFIA, NFIB, NFIC and NFIX, which are highly conserved from chicken to human. In vertebrates, NFI gene transcripts undergo alternative splicing so that multiple proteins can be formed out of each Nfi gene. Functional activities of these proteins generated from the four Nfi genes proved different characteristics. (Gronostajski, 2000).

NFI family members are identified as site-specific DNA binding proteins (Nagata et al., 1983). NFIs bind as a dimer to the consensus site TTGGC(N5)GCCAA on double-stranded DNA with high affinity (Kd~10−11 M), whereas they bind to individual half sites TTGGC or GCCAA with lower affinity (Kd~10−9 M) compared to the dyad symmetric sequence (Meisterernst et al., 1988a). The common NFI protein structure contains a highly conserved DNA binding N-terminal domain which is about 220 amino acids long and includes a putative lysine-rich helical region plus four conserved cysteine residues (Figure 1.1). One of the cysteine residues is employed in redox control, rendering NFI protein sensitive to oxidative inactivation. The remaining three cysteine residues are required in the DNA binding process (Bandyopadhyay and Gronostajski, 1994). N-terminal domain of NFI was not found to be homologous to known DNA binding domains (helix-turn-helix, helix-loop-helix, leucine zipper and zinc or ring finger). *In vitro*, the NFI N-terminus was shown to be sufficient for DNA binding, dimerization and the initiation of adenoviral replication. (Bandyopadhyay and Gronostajski, 1994).

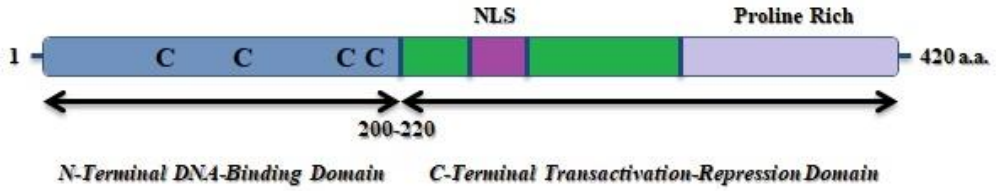

Figure 1.1: Structure of the NFI transcription factor. NFI harbors a DNA-binding domain and 4 Cysteine residues at the N-terminus. There is a transactivation-repression domain and a proline-rich domain at the C-terminus. A nuclear localization signal (NLS) is also included in the C-terminal site.

The C-terminal end of NFI proteins harbors a 100 residue domain which has high (%25) Proline content and is less conserved among NFI proteins compared to the N-terminal site (Figure 1.1) (Mermod et al, 1989). In insect and mammalian cells, the C-terminal Proline-rich domain was shown to be essential to fully activate transcription of an NFI-site containing promoter (Mermod et al, 1989). In addition to transcriptional activation, NFI binding sites may also promote transcriptional repression. A negative ragulatory domain was identified in NFIX (Nebl and Cato, 1995). In NFIA and NFIC, the repression activity is attributed to the proline rich domains (Osada et al., 1997b). Contrary to the similarity of peptide sequences of NFI family members, variation in the C-terminal domain resulting from alternative splicing and different overall posttranscriptional modifications, each NFI family member may have differential interactions with other proteins which may result in functional specificity. Mouse knockout studies show that the function of an individual NFI protein cannot be compensated by other NFI family members, suggesting that there are mechanisms specific to each NFI protein (Shu T. et al., 2003; Steele-Perkins G. et al., 2003; Steele-Perkins G. et al., 2005).

1.1.1 Role of NFI transcription factor in neural development

NFI transcription factors are primarily expressed by stem cells and progenitors in the ventricular zones within the central nervous system (CNS) (Mason et al., 2008). In situ hybridization experiments on mice show that *Nfia* mRNA expression occurs in cortex during both early and late development, while *Nfib* and *Nfix* are likewise highly expressed in the developing neocortex and midbrain. (Chaudhry et al., 1997). Malformations in the hippocampus (Piper et al. 2009, 2014; Heng et al. 2014) pons (Kumbasar et al. 2009), and spinal cord (Deneen et al. 2006) development are seen in mice that lack individual *Nfi* family members (Harris et al. 2015; Fraser et al., 2016).

Nfia$^{-/-}$, *Nfib*$^{-/-}$ or *Nfix*$^{-/-}$ mice display defects in development of corpus callosum (das Neves et al., 1999; Driller et al., 2007) and enlargement of lateral ventricles (Campbell et al., 2008).

Absence of *Nfia* expression results in late gestation neuroanatomical malformations such as reduced size of forebrain commissures, failure of corpus callosum development and loss of particular midline glia (Steele-Perkins G. et al., 2005). Conditional *Nfib*$^{-/-}$ cortex displayed loss of late basal progenitors and axonal projections of cortifugal neurons (Betancourt J. et al., 2013). Absence of NFIB caused a delay in the development of corpus callosum (Piper M. et al., 2009). In *Nfix* knockout studies, mice could survive until postnatal day 21 (P21) (Driller K. et al., 2007). *Nfi*$^{-/-}$ mice exhibited delays in the development of neurons and glia within the cerebellum (Fraser et al., 2016), retarded growth and defective corpus callosum and brain ventricles (Driller K. et al., 2007). *Nfix*$^{+/-}$ mice exhibited reduced NFIX expression in the brain (Driller K. et al., 2007). *Nfix*$^{+/-}$ mice exhibited disrupted hippocampal phenotype which persisted in the adults. Display of reduced performance by these mice in the Morris water maze learning and memory challenge suggest that the level of NFIX expression is critical for the development and function of the hippocampus (Harris L. et al., 2013). Absence of *Nfix* was shown to delay the production of intermediate progenitor cells (IPCs), causing an increased number of neurons in the postnatal forebrain (Harris L. et al., 2016). Loss of one more NFI allele, the *Nfib*, progressed the delay further. On the contrary, Nfix overexpression resulted in the generation of IPC prematurely (Harris L. et al., 2016). Moreover, Nfix nonsense mutation and microdeletion are associated with an overgrowth syndrome called the Malan syndrome (Dong Hai-Yun et al., 2016)

The chromatin-modifying protein Enhancer of zeste homolog 2 (EZH2) promotes neural progenitor cell self-renewal during cortical development. Transcription factor NFIB binds to EZH2 promoter region. In Nfib$^{-/-}$ mice, EZH2 is upregulated and EZH2 target genes are misregulated in neocortex and hippocampus whereas NFIB overexpression leads to decreased EZH2 transcription. Therefore, NFIB may regulate cortical progenitor differentiation through revoking EZH2 function (Piper M. et al., 2014).

In the hindbrain, cerebellar granule neuron terminal differentiation, synaptogenesis and dendritogenesis, NFIs regulate temporal gene expression through delayed

occupancy of target promoters (Ding B. et al., 2016). Also, NFI was shown to coordinate formation of dendrites, axonal growth and migration of CGNs (Wang et al., 2007). These data collectively imply essential functions for NFI members in the development of the nervous system.

1.1.2 Binding partners of NFI transcription factor family

NFI transcription factors interact with specific proteins and thereby control gene expression. Ski oncoprotein was shown to interact with NFI proteins, and in the presence of this interaction, Ski can bind to NFI binding sites, activating the transcription of a reporter gene (Tarapore et al., 1997). In addition, coimmunoprecipitation studies revealed that Regulatory Factor X (RFX) interacts with NF1 in a regulatory complex (Norquay et al., 2003). Moreover, NFIA interacts and forms a complex with the transcription factor Sox9 and through this relationship regulates the genes that are involved in the initiation of gliogenesis (Kang et al., 2012). Likewise, the transcription factors NFIA and Sox10 interact and antagonize the function of one another, promoting progenitor fate decision. Interaction with Sox10 prevents NFIA from inducing genes that are involved in astrocyte differentiation, while binding of NFIA to Sox10 inhibits oligodendrocyte differentiation by inhibiting Sox10 function (Glasgow et al., 2014).

NFI family members function in the control of postmitotic differentiation of central granule neurons (CGNs). One essential part of NFI functioning in this process is cell-to-cell signaling. Tag-1 is a cell adhesion molecule which is strongly expressed in CGNs that undergo parallel fiber formation. Downregulation of Tag-1 occurs before radial migration. In immature primary CGNs, NFI is involved in the repression of Tag-1 expression. This effect was also demonstrated in the cerebella of *Nfib*$^{-/-}$ mice at embryonic day 18 (E18). Transient transfection and functional *Nfi* knockout techniques followed by chromation immunoprecipitation in mice implicated that NFI directly regulates the Tag-1 gene, and Wnt7a, required for postmitotic mossy fiber CGN maturation, is directy targeted by NFI. (Wang W. et al., 2010).

Phenotypic divergence is linked to migration, proliferation and survival of tumor cells. Switching between invasive and proliferative behaviors was attributed to the heterogeneity of BRN2 and MITF transcription factors. NFI transcription factor which was determined as a downstream effector of BRN2 function, is associated

with this phenotypic switch, promoting invasive and migratory behavior in melanoma cells. Moreover, NFIB upregulates the epigenetic factor EZH2 which attenuates the expression of MITF, inducing melanoma cells to become less proliferative and more invasive (Fane et al., 2017).

Brg1 Associated Factor (BAF) complex enables transcription via ATP-dependent chromatin remodeling by virtue of its ATPase core subunit BRG1. Dual-immuno-gold labeling in HeLa cells showed that BRG1 is colocalized with both NFI and RNA polymerase II in the nucleus. Furthermore, interactions of BRG1, NFI and RNA polymerase II were confirmed by co-immunoprecipitation assay in HeLa cells and in activated mouse spleen T lymphocytes. The interaction of BRG1 with NFI was dependent on the activation of transcription in the lymphocytes and was not detected in the resting lymphocytes (Zhao L. et al 2005).

1.2 BAF Chromatin Remodeling Complex

Cells have their DNA tightly packaged into the compact form chromatin for convenient storage of large amounts of DNA. The chromatin is composed of building blocks called the nucleosome,. A nucleosome unit is defined by a 147 base pair length of DNA wrapped around an octamer of histone proteins. These proteins are called the four histones H2A, H2B, H3 and H4, and pairs of each together constitute the histone octamer (Luger et al. 1997; Kornberg R. 1974). Following the wrapped DNA, about 10-90 base pairs of spacer DNA exists and joins the unit with the next nucleosome. Further compaction of nucleosomes transforms the structure into heterochromatin which needs to be remodeled to the more loose form euchromatin to allow the activation or repression of genes localized to that nucleosomal site (Thoma et al., 1979; Widom and Klug, 1985). Chromatin remodeling is regulated by histone modifying enzymes (Wang Y et al., 2004) and ATP-dependent chromatin remodeling complexes (Saha et al., 2006; Clapier & Cairns, 2009). Histone-modifying enzymes recognize specific residues of histone tails and covalently mark them via modifications such as methylation, acetylation, phosphorylation, ribosylation or ubiquitination (Strahl and Allis, 2000). Specific recognition of histone marks by the ATP-dependent chromatin remodeling complexes provides ATP hydrolysis which can unwrap, mobilize, exchange or eject the nucleosome. Changes in the nucleosome structure leads to recruitment a transcriptional machinery to the

local DNA, and in this way the whole process enables regulation of gene expression (Tang et al. 2010).

BRG1/BRM Associated Factor (BAF) complexes are ATP-dependent chromatin-remodelers that alter chromatin structure via ATP hydrolysis (Figure 1.2). At least 15 subunits constitute the mammalian BAF complex. These molecules are similar to yeast SWItch/Sucrose Non-Fermentable (SWI/SNF) complexes with which they have five subunits in common (Cairns et al., 1994) (Figure 1.3). Hence, they are alternatively termed as mammalian SWI/SNF (mSWI/SNF) complexes (Son E.Y. & Crabtree G.R., 2014). The common subunits are named BRG1/BRM, BAF155/170, BAF60, BAF53, and BAF47 in the mammalian BAF complex. Apart from the yeast homolog of BAF complex, a homolog in Drosophila was discovered. This complex is called the Brahma-associated protein (BAP) complex and has the core ATPase subunit Brahma (BRM) (Tamkun et al., 1992).

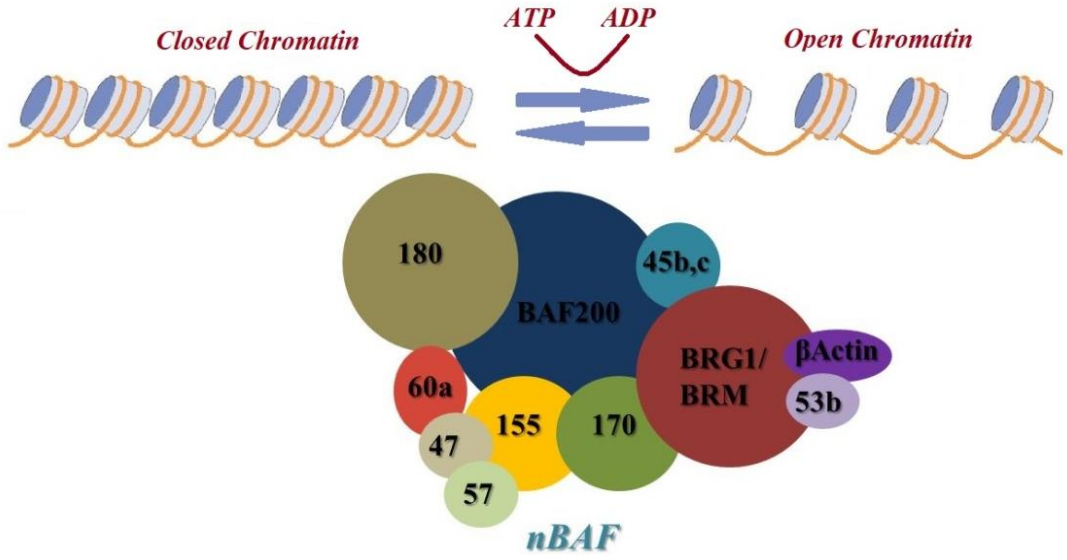

Figure 1.2: Altering of chromatin structure by the nBAF complex. All BAF complexes can open closed chromatin arrangements or close open ones using the Brg1/Brm ATPase subunit in their structures. Thereby it participates to the regulation of gene expression (Adapted from Yoo A. S. & Crabtree G.R., 2009)

BAF complex engages in multiple protein-protein and protein-DNA interactions. The interaction modules of the complex act together to remodel chromatin. BRG1/hBRM subunit exhibits ATPase activity due to enzymatic HELICc and DExx domains in its structure. QLQ domain is involved in binding to proteins (Kim et al, 2003). HSA domain facilitates interaction of BAF53 and β-actin (Szerlong et al., 2008). The bromodomain at the end provides BRG1 interaction with the acetylated histone tails

(Haynes et al., 1992). The function of the BRK domain has not been elucidated. BAF155 and BAF170 are scaffold proteins for the complex (Chen and Archer, 2005). Additional domains may provide specific interactions with other proteins or DNA. (Figure 1.3).

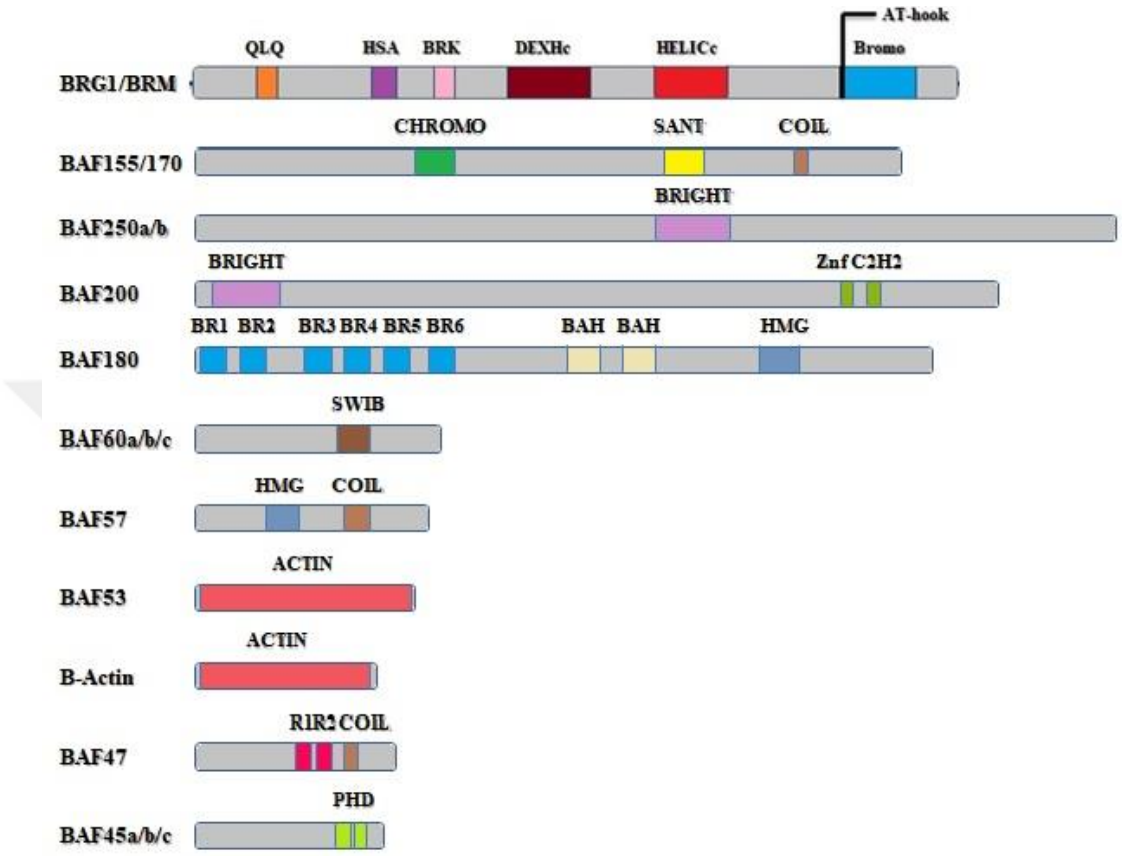

Figure 1.3: Structures of different subunits of BAF complex (Adapted from Tang et al., 2010).

Many of the positions in the BAF complex can be occupied by alternative subunits. Therefore, different combinations of these exchangeable subunits would potentially generate a diverse set of BAF complexes. However, once assembled, the final BAF complex is quite stable, the subunits remain bound to the complex even in high denaturing environment, in vitro. (Zhao et al., 1998; Kadoch et al., 2013). Unique BAF assemblies have been reported to be expressed in a tissue-specific manner and to interact with many transcription factors in different cell types (Ho and Crabtree et al, 2010). According to the cellular context, specific subunits are assembled into the neural subtype-specific BAF complexes and constitute unique assemblies in neural progenitors (npBAF), neurons (nBAF), layer 5 neurons (l5-nBAF), oligodendrocytes (olBAF) and Schwann cells (scBAF) (Narayanan and Tuoc, 2014). Due to

combinatorial assembly of their subunits, BAF complexes have dedicated functions at various stages of neural differentiation, during which they control the generation of distinct neural cell types and the modulation of trans-differentiation between cell types. Besides, tight interactions between the transcriptional machinery and the BAF complex were shown to regulate differentiation of NSCs (Narayanan and Tuoc, 2014).

1.2.1 Role of mammalian BAF complexes in neural development

The generation of neural subtypes from neural progenitors occurs through the activation and/or repression of several transcriptional programs (Wen S. et al., 2009; Muhchyi C. et al., 2013; Ronan J.L. et al., 2013). Epigenetic modifications and chromatin regulation are responsible for the regulation of transcriptional programs. These processes generate persistent changes in the chromatin state both by causing direct effects on gene transcription and by creating platforms for the recruitment of transcription factors (TFs) (Wen S. et al., 2009; Muhchyi C. et al., 2013; Ronan J.L. et al., 2013). Regulation of gene expression by tissue specific transcription factors in coordination with distinct BAF complexes may imply the extensive function of BAF complexes in development of nervous system, in addition to many other organs (Zhan et al., 2011; Weider et al., 2012; Ninkovic et al., 2013).

Studies of mammalian BAF complexes revealed that the combination of the subunits is progressively altered during the transition from a pluripotent stem cell to a multipotent neuronal progenitor cell to a committed neuron (Crabtree et al, 2010). Figure 1.4 shows the transformation of the BAF complex in neural progenitors (designated as npBAF) into the BAF complex in neurons (designated as nBAF). In neural progenitors the two subunits BAF45a and BAF53a are assembled into the npBAF complex. The expression of these two subunits is reduced in the cells as they are replaced by homologous subunits during neuronal differentiation (Yoo A.S., & Crabtree G.R., 2009). Reduced proliferation of neural progenitors with the inhibiton of BAF45a or BAF53a, and also increased proliferation obtained by the overexpression of BAF45a indicate that the npBAF complex is critical in controlling the self-renewal of neural progenitors (Yoo A.S., & Crabtree G.R., 2009).

Overexpression of BAF60c, as with BAF45a and BAF53a, promoted proliferation of neural progenitors. In addition, BAF60c was found to be absent in differentiated

neurons in the developing brain and in the neural tubes of mouse embryos (Takeuchi J.K. et al., 2007). Therefore, BAF60c is also proposed as a neural progenitor BAF subunit.

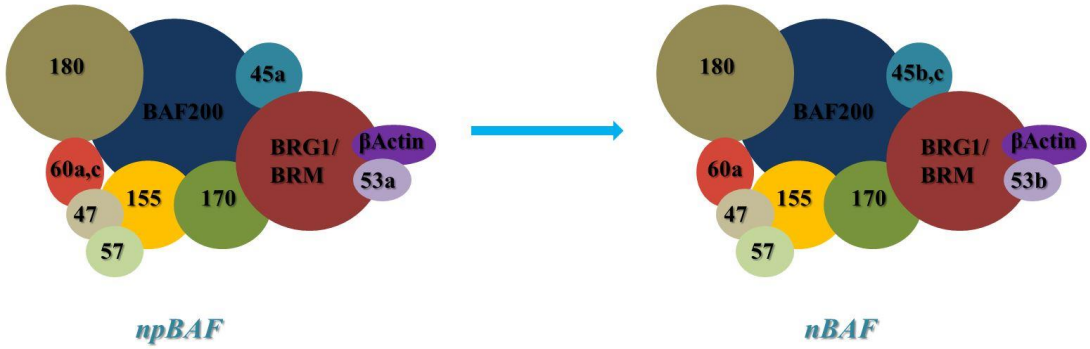

Figure 1.4: Transformation of the neural progenitor BAF complex (npBAF) into the neuron-specific BAF complex (nBAF). The npBAF complex in neural progenitor cells is transformed into nBAF complex in neurons as BAF45a and BAF53a subunits are exchanged for BAF45b,c and BAF53b (Adapted from Yo A.S. & Crabtree G.R., 2009).

Three subunits are dedicated to be incorporated into the neuron-specific BAF complex (nBAF): BAF53b, BAF45b, and BAF45c. In neuronal development, the exchange of BAF53b and BAF45b,c subunits with their non-neuronal counterparts is a critical regulatory switch, the final step in neurogenesis (Olave et al., 2002; Lessard et al., 2007; Hargreaves and Crabtree, 2011). Repression of BAF53a expression and upregulation of BAF53b, BAF45b and BAF45c expression is essential for neuronal cell fate. Neuronal differentiation is prevented both by the inhibition of BAF53b expression or by the maintenance of BAF53a expression. The conversion from BAF53a to BAF53b was also found to have implications for adult cognition (Hargreaves and Crabtree, 2011; Lopez et al 2015). The final combinatorial assembly of BAF complexes was has been known to play an essential role in neurodevelopment; however, recent reports suggest that it also is involved in cognitive functions in the adult human brain (Yoo A.S. et al., 2009; Choi et al., 2015).

1.3 Brg1/BAF190 (Brg1 Associated Factor 190)

The mammalian BAF complex includes an ATPase subunit called BRG1 (Brahma-related gene 1) one of the 12 non-exchangeable subunits *in vitro*. BRG1 comprises multiple domains (Figure 1.5). The catalytic ATPase domain is conserved and lies in

the middle. At the C-terminal region there is a conserved bromodomain and an AT-hook motif whereas the N-terminal end carries QLQ, HSA and BRK domains [Fan et al., 2005; Khavari et al., 1993].

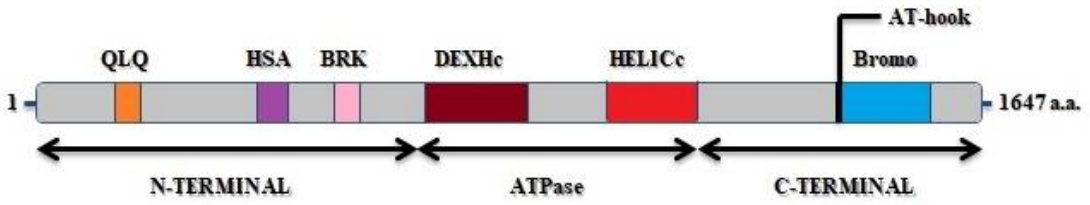

Figure 1.5: Structure of the BRG1 protein. BRG1 harbors QLQ, HSA and BRK domains at the N-terminus. The DEXHc and HELICc domains in the structure are ATPase domains. At the C-terminus, there is a AT-hook motif and a Bromo domain (Adapted from Tang et al., 2010).

The DEXHc domain that contains the ATP-binding region is related to DEXH-box helicases whose function is to unwind nucleic acids in the cell. HELICc domain is also associated with a number of helicase-like proteins. Helicases unwind the nucleic acids using the free energy liberated from nucleoside triphosphate hydrolysis (Trotter et al., 2008).

Histone modifications within a promoter may provide an interaction site for regulators containing bromodomains (Singh et al., 2006). The bromodomain at the C-terminal end of BRG1 was considered to be recognizing the acetylated lysine residues on histone tails. (Chandrasekaran and Thompson, 2007; Shen et al., 2007). The AT-hook motif at the C-terminus of BRG1 may be also involved in DNA binding and recruitment to acetylated histones (Trotter et al., 2008).

The functional importance of QLQ (glutamine-leucine-glutamine) motif at the N-terminal end of BRG1 (located between amino acids 172-208) has not been determined. However, it may be involved in protein-protein interactions or facilitate protein folding (Kim et al., 2003; Williamson, 1994). The HSA domain identified within the amino acids 475-532 is also present in helicases, yet its role is yet to be discovered (Doerks et al., 2002). The function of BRK domain at amino acids 612-656 is likewise unidentified. Also known as TCH domains, BRK domains are linked to transcription and chromodomain helicases (Allen et al., 2007; Doerks et al., 2002). Although their functions are not well studied or unknown, domains at the N-terminal end of BRG1 may be important for its activity (Trotter et al., 2008).

1.4 Role of BRG1/BAF190 in neural development

BRG1 protein is required for neural development, specifically for neural stem cell self-renewal and neurogenesis. Notch and Sonic hedgehog signalling pathways are the key pathways in regulation of neurogenesis. Absence of BRG1 in neural progenitor cells results in misexpression of central components in these pathways, showing that BRG1 is required for proper neuronal differentiation (Lessard J. et al, 2007). Knockdown of *Brahma* (a Brg1/Brm homolog) leads to defective dendrite morphogenesis in Drosophila embryos. (Yoo A.S. & Crabtree G.R., 2009). *Brg1* conditional knockout in neural progenitor cells of higher vertebrates were not able to perform self-renewal, leading to a reduction in the number of total neural progenitor cells. Since the fibroblast population was not affeced in the *Brg1* knockout, the phenotype appeared to be limited to neural progenitor cells only (Yoo A.S. & Crabtree G.R., 2009).

Interestingly, loss of Brm, a mammalian Brg1 homolog, did not produce any significant change in the neural phenotype (Reyes et al., 1998), whereas Brg1 deficient mice died before or during implantation (Bultman et al., 2000). In addition, abnormal neural tube formations were detected in *Brg1*$^{-/-}$ mice in later stages of embryonic development. Hence, Brg1 performs more important functions than its homolog Brm in neural development (Bultman et al., 2000).

Brg1 was also shown to have important functions in the postnatal neural development. In the CNS of conditional *Brg1* knockout mice, obtained by Cre-Lox recombination, the body and brain sizes were severely diminished at postnatal day 35 (p35). *Brg1* knockout also resulted in serious reduction in the number of cortical neurons and their dendritic branches during early postnatal development. (Deng et al., 2015).

Brg1 also plays a role in development of cancer in neural cells. Neuroblastoma is a sympathetic nervous system tumor that is resistant to many treatments. Recently, elevated levels of BRG1 expression was detected in later stages of neuroblastoma. Loss of function experiments both *in vitro* and *in vivo* suggested that BRG1 is required for the proliferation of neuroblastoma cells (Jubierre et al., 2016).

During the development of the peripheral nervous system, Brg1 protein is associated with the differentation of murine Schwann cells and their myelination *in vitro* and *in*

vivo. Schwann cells are stimulated by the signals from the axons so that myelin genes become upregulated and the cell cycle is arrested. BRG1 activity was found to be high in Schwann cells early in myelination, while loss of BRG1 constrained Schwann cell differentiation and eliminated myelination (Limpert et al., 2013).

Activation of BRG1 is necessary and sufficient for the initiation of oligodendrogenesis and maturation of oligodendrocytes (Yu et al., 2013). Olig2 was shown to interact with and regulate the activity of a BRG1-dependent chromatin remodeling complex through its BRG1 subunit in order to promote oligodendrocyte differentiation and myelination throughout the central nervous system (Yu et al., 2013).

In addition to neural cell differentiation, BRG1 is associated with neural stem cell maintenance. BRG1 activity was shown to prevent commitment of neural stem cells to neural fate by maintaining NSCs sensitive to gliogenic signals. Ventricular zone NSCs of conditional *Brg1* knockout mice prematurely differentiated into post-mitotic neurons prior to initiation of gliogenesis, causing a powerful decline in the number of oligodendrocytes and astrocytes. Growth-factor-induced differentiation by gliogenic progenitors into astrocytes *in vitro* was also inhibited by the removal of *Brg1*. In *Brg1* mutant mice the levels of Sox1, Pax6 and Musashi-1 proteins which act in stem cell maintenance were decreased significantly in the ventricular zone. These evidences collectively implicated that the switch from neurogenesis to gliogenesis was regulated by BRG1 (Matsumoto et al., 2006).

1.5 Binding partners of BRG1/BAF190

BRG1 is an enzymatic subunit of the chromatin remodeling BAF complexes in which it serves as the main ATP hydrolysis unit. Through the protein-protein interaction sites present in its structure, BRG1 interacts with a number of transcription factors to regulate chromatin structure and gene expression during development. In vertebrate peripheral nervous system, terminal differentiation of Schwann cells requires BRG1 (Smarca4) which is recruited by Sox10 transcription factor during the myelination activity (Bischof et al., 2015). During the development of trophoblast in mice in preimplantation development, the BRG1 subunit interacts

with Histone deacetylase 1 (HDAC1) enzyme to provide the necessary Nanog suppression (Carey et al., 2015).

BRG1 interacts with pioneer transcription factors in order to control pluripotency network. During developmental processes and cellular reprogramming, the pioneer transcription factors bind to inaccessible chromatin sites containing target sequences and designate these regions open to transcription. OCT4 is one such pioneer molecule that creates open chromatin structures via binding to other transcription factors and to chromatin remodeling factors. BRG1 interacts with OCT4 and participate in altering target chromatin structure, playing important roles in cellular reprogramming and development (King H.W. & Klose R.J., 2017).

Proteins that together constitute a chromatin remodeling complex can also affect the expression of one another. Two proteins within the chromatin remodeler BAF complex, SMARCAL1 and BRG1, were shown to bind to each other's promoter sites and increase their expression in order to promote DNA repair in case of DNA damage (Dominic et al., 2016).

Smad3 is yet another transcription factor that BRG1 binds to. *In vitro* experiments revealed an interaction between BRG1 and Smad3. Moreover, *in vivo* studies indicated that BRG1 and endogenous Smad3 interaction was enhanced by TGFβ stimulation which causes influx of Smad proteins in the nucleus where BRG1 is located. The transcription complex formed by this interaction controls the transcription of the target gene (Massague et al., 2007). Likewise, BRG1 interacts with the transcription factor NFI and with the RNA polymerase II in a transcriptional assembly (Zhao L. et al 2005).

Another protein that was suggested in literature to interact with BRG1 is Geminin. BRG1 functions in cell cycle exit and subsequent cellular differentiation. During neurogenesis, Geminin interacts with Brg1 to interfere with its activity and keep the cell in the undifferentiated state (Kroll et al., 2005).

BRG1 was also shown to have a function in cardiomyogenesis. The Hedgehog signaling pathway controls the differentiation of mouse embryonic stem cells into cardiac progenitor cells. Blocking of this signaling pathway attenuates the differentiation into cardiac progenitors. Overexpression of the transcription factor GLI2 upregulates the cardiac progenitor-enriched Mef2. Recently, it was shown *in*

vitro that BRG1 interacts with GLI2 to enhance the expression from Mef2c gene in P19 mouse embryonic stem cells during cardiomyogenesis and controls the Hedgehog-dependent cardiomyogenesis in this manner (Fair et al., 2016).

As well as activation of gene expression, BRG1 was also associated with repression of target gene expression. It was suggested that the BRG1 protein interacts with the transcription factor REST (Repressor Element 1-Silencing Transcription factor) and facilitates the silencing of the REST's target genes (Ooi et al., 2006).

1.6 Aim of Study

BAF complex is a chromatin remodeling factor which harbors the BRG1 ATPase enzymatic subunit in its structure which uses ATP to alter the structure of chromatin (Son, E. Y. & Crabtree, G. R., 2014). BAF complexes that include BRG1 subunit are present in neural progenitors and neurons (Narayanan, R. & Tuoc, T. C., 2014). BAF complexes play various roles in developmental processes through the transcription factors it interacts (Ronan et al., 2013).

Nuclear Factor I is a transcription factor that has four different isoforms (NFIA, NFIB, NFIC and NFIX) and is expressed throughout the human brain during development and in the adult. NFI transcription factor was found to interact with BRG1 in HeLa cells and through this interaction is involved in activation of transcription in T lymphocytes (Zhao L. et al, 2005). Zhao L. et al did not investigate the NFI family member with which BRG1 interacts or the domains involved in this interaction. Therefore, we set out to recapitulate this interaction in neural cell line, SH-SY5Y neuroblastome cells as well as overexpression in HEK293T cells to investigate the if BRG1-NFI binding is NFI family member and BRG1 domain specific. Elucidating BRG1 and NFI interaction will contribute to our understanding of how these two proteins regulate gene expression during neural development.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

Chemicals that were used in the experiments performed throughout the study are listed in Table 2.1.

Table 2.1 : Chemicals that were used in the experiments.

Chemical	Supplier	Catalog Number
0.05% Trypsin-EDTA 1X, Phenol Red	Gibco	25300054
Absolute ethanol	Carlo Erba	4146082
Acrylamide (40%)	Applichem	A4989
Agar	Labm	LABMC006
Agarose	Seakem LE	50004
Ammonium persulfate	Fisher Scientific	BP179-100
Bromophenol Blue	Fisher Scientific	BP115
Dimethyl sulfoxide (DMSO)	Fisher Scientific	BP231
DNA Loading Dye (6X)	Thermo Scientific	R0621
Dulbecco's Modified Eagle Medium w/o FBS, L-Glutamine	Lonza	BE12-614F
DPBS w/o calcium, magnesium	PAN	P04-36500
DTT		
Ethylenediamine tetraacetic acid	Fisher Scientific	BP118
Fetal Bovine Serum	Gibco	10270106
Glycerol	MP	800689
Glycine	Neofroxx	50-40-6
Hydrochloric acid (37%)	Carlo Erba	403872
L-Glutamine (200 mM, 100X)	Gibco	25030-024
Methanol	Sigma Aldrich	24229
Non-fat dry milk	Cell Signalling	9999
Nonidet P40	Applichem	A1694
Penicillin/Streptomycin	Gibco	15140-122
PBS w/o calcium, magnesium	Lonza	BE17-516F
Ponceau-S	Fisher Scientific	BP103
Pronasafe nucleic acid staining dye	Conda Lab	CK130
Protease Inhibitor Cocktail (Pi)	ThermoFisher	78430
SDS	MP	194831
Sodium chloride	Merck	M106404
Teksoll (96%) extra pure	Tekkim	TK.200650.25001

Table 2.1 (cont'd) : Chemicals that were used in the experiments.

Chemical	Supplier	Catalog Number
Temed	Sigma	T7024
Tris base	Fisher Scientific	BP152-1
Trizma Base	Sigma	T6066
Tryptone	Labm	MC005
Tween 20	Fisher Scientific	C58H114O26
Yeast Extract	Labm	MC001

2.1.2 Antibodies

Antibodies that were used in western blot and co-immunoprecipitation experiments are given in Table 2.2.

Table 2.2 : Antibodies that were used in the experiments.

Antibodies	Supplier	Catalog Number	Concentration
Polyclonal Flag Tag Antibody Produced in Rabbit	CST	2368	1:1000
Anti-HA Antibody Monoclonal Produced in Mouse	Roche	12CA5	1:500
Anti-HA Tag Monoclonal Antibody Produced in Rabbit	CST	3724	1:1000
Stabilized Peroxidase Conjugated Goat Anti-Rabbit (H+L)	Thermo Scientific	32460	1:1000
Stabilized Peroxidase Conjugated Goat Anti-Mouse (H+L)	Thermo Scientific	32430	1:1000
Anti-NFIB Antibody Produced in Rabbit	Sigma-Aldrich	HPA003956	1:2000
Anti-Brg1 Antibody Produced in Mouse	Santa Cruz Biotechnology	Sc-17796	1:1000
Mouse IgG	Santa Cruz Biotechnology	Sc-2025	-
Veriblot	Abcam	131366	1:1000

2.1.3 Media

2.1.3.1 Mammalian cell culture media

Complete growth medium for HEK293T cell culture was prepared by mixing 450 ml of Dulbecco's Modified Eagle's Medium (DMEM), 50 ml of Fetal Bovine Serum

(FBS), 5 ml of L-Glutamine and 5 ml of Penicillin/Streptomycin antibiotics. Growth media were stored at +4^{o}C.

Freezing medium for HEK293T cells was prepared by mixing 5 ml of DMEM, 4 ml of FBS and 1 ml of DMSO. Freezing media were stored at -20^{o}C.

Antibiotic-free growth medium that was used in the transfection experiments with PEI Branched was prepared by mixing 44.5 ml of DMEM, 5 ml of FBS and 0.5 ml of L-Glutamine. Antibiotic-free growth media were stored at +4^{o}C.

2.1.3.2 Bacterial cell culture media

LB agar medium was prepared by dissolving 15 g of NaCl, 5 g of yeast extract, 10 g of tryptone and 5 g of agar in 900 ml distilled water by stirring. Then, the total volume was brought to 1 L with distilled water. The medium was sterilized by autoclaving at 120^{o}C for 15 min and stored at +4^{o}C.

LB medium was prepared by dissolving 10 g of NaCl, 5 g of yeast extract and 10 g of trptone in 900 ml distilled water by stirring. The total volume was brought to 1 L with distilled water. The medium was sterilized by autoclaving at 120^{o}C for 15 min and stored at +4^{o}C condition.

NZY^{+} medium was prepared by dissolving 10 g of NZ amine, 5 g of yeast extract, 5 g of NaCI in deionized water up to the final volume of 1 L. pH was adjusted to 7.5 using NaOH and the medium was sterilized by autoclaving at 120^{o}C for 15 min and by filtering. 12.5 ml of 1 M $MgCl_2$, 12.5 ml of 1 M $MgSO_4$ and 20 ml of 20 % (w/v) glucose were added into the sterilized medium under sterile conditions with the help of a bunsen burner. The medium was stored at +4^{o}C.

2.1.4 Plasmids

pCMV5 Brg1-Flag, pCMV5 BAF170-Flag, pMYC Brg1 aa597-1407 and pMYC Brg1 aa1400-1648 plasmids (Addgene plasmid #19143, #19142, #19146 and #19145 respectively) were used in this study. The plasmids were a gift from Joan Massague Laboratory located in Memorial Sloan Kettering Cancer Center, New York, USA. pcDNA3.1 HA-ATXN3 and pcDNA3.1 FLAG-MAD2 plasmids were previously designed in our laboratory (Yılmazyıldırım E., 2017).

2.1.5 Restriction enzymes and buffers

Restriction enzymes and buffers used in the restriction enxyme digestion experiments are displayed in Table 2.3.

Table 2.3 : Enzymes and buffers that were used in the experiments.

Restriction Enzymes	Supplier	Catalog Number
SalI-HF	New England BioLabs	R3138S
EcoRI-HF	New England BioLabs	R3101S
HindIII-HF	New England BioLabs	R3104S
Xbal	New England BioLabs	R0145S
Ncol	New England BioLabs	R0193S
10X 3.1 Buffer	New England BioLabs	B7203
10X CutSmart Buffer	New England BioLabs	B7204
MassRuler DNA Ladder Mix	Thermo Scientific	SM0403
Protein Pre-stained Marker	New England BioLabs	P7712

2.1.6 Commercial kits

Commercial kits that were used in the Maxiprep plasmid isolation, determination of protein concentration and western blot experiments are listed in Table 2.4.

Table 2.4 : Commercial kits that were used in the experiments.

Commercial Kits	Supplier	Catalog Number
GeneJet Plasmid Maxiprep	Thermo Scientific	K0492
Pierce BCA Protein Assay Kit	Thermo Scientific	23227
Western Bright ECL	Advansa	K12045
In-Fusion HD Cloning Kit	Clontech	011614
CloneAmp HiFi PCR Premix	Clontech	639298
NucleoSpin Gel and PCR Clean-Up Kit	Macherey-Nagel	740609

2.1.7 Transfection and lysis solutions

2.1.7.1 PEI Linear solution

Polyethylenimine (PEI) Linear transfection reagent was prepared by dissolving PEI Linear powder in distilled water and adjusting the pH to 7.0 with HCl until the solution became clear. Final concentration was adjusted to 1 mg/ml and the final solution was filtered through a 0.22 μm membrane for sterilization. The filtered solution was stored as aliquots at -80^{o}C condition. Once thawed, aliquots were further stored at +4^{o}C.

2.1.7.2 1X SDS sample buffer

To prepare 10 ml 1X SDS sample buffer, 1.25 ml of 0.5 M Tris-HCl (pH: 6.8) (final concentration: 62.5 mM), 2 ml of %10 (w/v) SDS (final concentration: %2 w/v) and 1 ml of Glycerol (final concentration: %10) were mixed. The final volume was adjusted to 10 ml with distilled water. 1X SDS sample buffer was stored at +4^{o}C.

2.1.7.3 1.5X SDS sample buffer

1.5X SDS sample buffer was used in order to elute the proteins which are bound to affinity resin. To prepare 1.5X SDS sample buffer, 150 µl of 100% Glycerol (final concentration: 15% v/v), 187.5 µl of 0.5 M Tris-HCl (final concentration: 93.75mM), 300 µl of 10% SDS (final concentration: 3% w/v), 75 µl of BromoPhenol Blue (10 mg/ml) (final concentration: 0.075 mg/ml) and 287.5 µl of dH_2O were mixed. The buffer was stored at -20^{o}C.

2.1.7.4 5X SDS sample buffer

5X SDS sample buffer was used in order to detect the protein expression. To prepare 5X SDS sample buffer, 600 µl of 0.5 M Tris-HCl (pH: 6.8) (final concentration: 300 mM) and 600 µl of 100% Glycerol (final concentration: 40% w/v) were mixed. Then, 0.1 g of SDS (final concentration: 0.01% w/v) and 0.1 g of BromoPhenol Blue (final concentration: 0.01% w/v) were added to the mixture. The buffer was stored at -20^{o}C.

2.1.7.5 NP-40 lysis buffer

To investigate protein-protein interactions, NP-40 lysis buffer was used in lysis of cells. To prepare NP-40 lysis buffer, 10 ml of 1 M Tris-HCl (pH: 7.5) (final concentration: 50 mM), 2 ml of NP-40 (final concentration: 1% v/v), 6 ml of 5 M NaCl (final concentration: 150 mM), 2 ml of 0.5 M EDTA (final concentration: 5 mM) were mixed. Final volume was adjusted to 200 ml with distilled water. The buffer was stored at +4^{o}C.

2.1.7.6 NEB-A and NEB-C buffer

To investigate endogenous nuclear protein-protein interactions, NEB-A lysis buffer was used in the lysis of cellular membrane and NEB-C lysis buffer was used in the

lysis of nuclear membrane. To prepare NEB-A lysis buffer, 50 µl of 1 M HEPES (pH: 7.9) (final concentration: 10 mM), 10 µl of 5 M NaCl (final concentration: 10 mM), 1 µl of EDTA (final concentration: 0.1 mM), 1 µl of EGTA (final concentration: 0.1 mM) were mixed. The final volume was adjusted to 5 ml with distilled water. To prepare NEB-C lysis buffer, 100 µl of 1 M HEPES (pH: 7.9) (final concentration: 20 mM), 400 µl of 5 M NaCl (final concentration: 0.4 M), 10 µl of EDTA (final concentration: 1 mM), and 10 µl of EGTA (final concentration: 1 mM) were mixed. The final volume was adjusted to 5 ml by adding distilled water. NEB-A and NEB-C buffers were stored for further use at +4^{o}C.

2.1.8 Western blot solutions

2.1.8.1 1M Dithiothreitol (DTT)

To prepare 1 M DTT, 1.54 g of DTT (molecular weight: 154.253 g/mol) was dissolved in total 10 ml distilled water. The solution was stored as aliquots at -20^{o}C condition.

2.1.8.2 10% Ammonium persulfate (APS)

To prepare 10% ammonium persulfate (APS), 1 g of ammonium persulfate was dissolved in 10 ml distilled water. The solution was stored as aliquots at -20^{o}C condition.

2.1.8.3 1.5 M Tris-HCl (pH: 8.8)

To prepare 1.5 M Tris-HCl (pH: 8.8), 90.825 g of Tris base (molecular weight: 121.14 g/mol) was dissolved in 400 ml distilled water. Then, HCl was added until pH reached 8.8. Afterwards, the final volume of the solution was brought to 500 ml by adding the required amount of distilled water. The solution was stored at room temperature.

2.1.8.4 1 M Tris-HCl (pH: 7.5)

To prepare 1 M Tris-HCl (pH: 7.5), 121.1 g of Tris base (molecular weight: 121.14 g/mol) was dissolved in 800 ml distilled water. Then, HCl was added until pH reached 7.5. Afterwards, the final volume of the solution was brought to 1 L by

adding the required amount of distilled water. The solution was stored at room temperature.

2.1.8.5 0.5 M Tris-HCl (pH: 6.8)

To prepare 0.5 M Tris-HCl (pH: 6.8) buffer, 18.15 g of Tris base powder (molecular weight: 121.14 g/mol) was dissolved in 200 ml distilled water and then, HCl was added until pH reached 6.8. Afterwards, the final volume of the solution was brought to 300 ml by adding the required amount of distilled water. The solution was stored at room temperature.

2.1.8.6 10% Sodium dodecyl sulfate (SDS)

To prepare 10% Sodium dodecyl sulfate (SDS) buffer, 30 g of SDS was dissolved in 300 ml distilled water. The solution was stored at room temperature.

2.1.8.7 0.1% Sodium dodecyl sulfate (SDS)

To prepare 0.1% SDS buffer, 1 ml of 10% SDS solution was added to 100 ml distilled water. The solution was stored at room temperature.

2.1.8.8 10X Running buffer

To prepare 10X Running buffer, 144 g of Glycine powder (molecular weight: 75.06 g/mol) and 30.2 g of Tris base (molecular weight: 121.14 g/mol) powder were dissolved in 800 ml distilled water. The volume of the buffer was brought to 1 L with distilled water. The solution was stored at room temperature.

2.1.8.9 1X Running buffer

To prepare 1X Running buffer, 100 ml of 10X Running buffer and 10 ml of 10% SDS were mixed. Then, the volume of the buffer was brought to 1 L with distilled water. The solution was stored at room temperature.

2.1.8.10 1X Transfer buffer

To prepare 1X Transfer buffer, 100 ml of 10X Running buffer and 200 ml of Methanol were mixed. Then, the volume of the buffer was brought to 1 L by adding the required amount of distilled water. The solution was stored at $+4^{o}$C.

2.1.8.11 1X TBS

To prepare 1X TBS, 50 ml of 1 M Tris-HCl (pH: 7.5) (final concentration: 50 mM), 30 ml of 5 M NaCl (final concentration: 150 mM) were mixed. Then, the volume of the buffer was brought to 1 L with distilled water. The solution was stored at +4^{o}C.

2.1.8.12 1X TBS-T

To prepare 1X TBS, 50 ml of 1 M Tris-HCl (pH: 7.5) (final concentration: 50 mM), 30 ml of 5 M NaCl (final concentration: 150 mM) and 1 ml of Tween 20 (final concentration: 0.1% v/v) were mixed. Then, the volume of the buffer was brought to 1 L with distilled water. The solution was stored at +4^{o}C.

2.1.8.13 5 M NaCl

To prepare 5 M NaCl, 58.44 g of NaCl (molecular weight: 58.44 g/mol) was dissolved in 160 ml of distilled water. Then, the volume of the solution was brought to 200 ml with distilled water. The solution was stored at room temperature.

2.1.8.14 Blocking buffer

Blocking solution was used to prevent non-specific antibody binding to the nitrocellulose membrane. To prepare blocking buffer, 1.5 g of non fat dry milk powder was dissolved in 50 ml of TBS-T. Blocking buffer was prepared fresh each time before use.

2.1.8.15 Ponceau-S solution

Ponceau-S solution was used to stain nitrocellulose membrane to check the efficiency of protein transfer to the membrane. To prepare Ponceau-S solution, 0.5 g of Ponceau-S (final concentration: 0.5% w/v) was dissolved in 1 ml acetic acid (final concentration: 1% v/v). Then, the volume of the solution was brought to 100 ml with distilled water. The solution was stored at room temperature.

2.1.8.16 SDS polyacrylamide gel

SDS polyacrylamide gel consists of stacking and separating gels of which compositions are explained in Table 2.5.

Table 2.5: Composition of SDS polyacrylamide gel.

	Separating gel				Stacking gel
	7.5%	**10%**	**12%**		**4%**
Acrylamide solution (40%)	1.88 ml	2.5 ml	3 ml	Acrylamide solution (40%)	0.5 ml
1.5 M Tris-HCl (pH: 8.8)	2.5 ml	2.5 ml	2.5 ml	0.5 M Tris-HCl (pH: 6.8)	1.25 ml
dH_2O	5.62 ml	4.9 ml	4.5 ml	dH_2O	3.2 ml
SDS (10%)	100 µl	100 µl	100 µl	SDS (10%)	50 µl
APS (10%)	100 µl	100 µl	100 µl	APS (10%)	50 µl
Temed	5 µl	4 µl	4 µl	Temed	5 µl

2.1.9 Agarose gel electrophoresis solutions

2.1.9.1 50X TAE

50X TAE was prepared by dissolving 242 g of Tris base (molecular weight: 121.14 g/mol) in 750 ml of distilled water and adding 100 ml of 0.5 M (pH 8.0) EDTA and 57.1 ml of glacial acetic acid. The final volume was set to 1 L by addition of distilled water. Dilution to 1X TAE was also made by addition of distilled water.

2.1.10 Laboratory equipment

Laboratory equipment that was used in the experiments are shown in Table 2.6.

Table 2.6 : Equipment that was used in the experiments.

Equipment	Supplier
Agarose Gel Electrophoresis System	BIORAD, Sub-Cell GT Agarose Gel Electrophoresis System
Autoclave	Zealway, GR
Benchtop Microcentrifuge	VWR Microstar 17R
Cell Culture Centrifuge	Harmony, LMS Group Thermo IEC CL10
Chemidoc	BIORAD, Chemidoc TM MP System
Falcon Tubes (15 ml, 50 ml)	Sarstedt, Nest, VWR
Freezer (-80^{o}C)	Panasonic
Freezer (-20^{o}C)	Bosch, GSD30N12NE
Gel Blot Paper	GB003
Hemocytometer	Marienfeld Neubauer improved
Incubator	Binder, CB150
Laminar Air Flow Cabinets	FASTER BH-EN 2003
Microcentrifuge Tubes (0.2 ml, 0.6 ml, 1.5 ml, 2 ml)	Axygen, Nest
Micropipettes (10 µl, 20 µl, 200 µl, 1 ml)	Gilson, Socorex

Table 2.6 (cont'd) : Equipment that was used in the experiments.

Equipment	Supplier
Microwave	Altus
Magnetic Stirrer	VWR, VS-C77, Wisd, MSH-20A
Microplate Reader	BIORAD
Microscope	Olympus, M021
Nanodrop	Thermofisher Scientific
Nitrocellulose Membrane	Santa Cruz, Ultracruz sc3724
pH meter	Inolab, pH720
Pipettor	CAPP
Power Supply	BIORAD
Rotator	Stuart, SB3, Stuart, STR4
Shaker	Heidolph, Duomax 1030
Spectrophotometer	Shimadzu, UV-1700
Thermoshaker	Biometra, TS1
Vortex	Heidolph, ReaxTop
Water Bath	Elektromag, M96KP Memmert, WB110

2.2 Methods

2.2.1 Bacterial growth for plasmid isolation

Luria-Bertani (LB) agar plates containing Ampicillin were inoculated with commercial bacteria containing the following overexpression plasmids: pCMV5 Brg1-Flag, pCMV5 BAF170-Flag, pMYC Brg1 aa597-1407 and pMYC Brg1 aa1400-1648. Bacterial cultures on agar plates were incubated at 37^{o}C for 16 hours. Single colonies were selected from LB agar plates and added to 5 ml of LB medium containing Ampicillin. Bacterial cultures in LB media were rotated at 200-225 rpm and 37^{o}C for 16 hours. After incubation, starter cultures were prepared for Maxiprep plasmid isolation. To this aim, 5 ml of each bacterial culture was diluted 1:1000-1:10000 in a total of 250 ml LB medium containing Ampicillin. Then, cultures were incubated at 37^{o}C for 16 hours at 200-225 rpm.

2.2.2 Maxiprep plasmid DNA isolation

Maxiprep plasmid DNA isolation was performed in order to isolate target plasmids from the bacteria that were grown in LB medium. Thermo Scientific Gene JET Plasmid Maxiprep Kit (#K0491, #K0492) was used in the isolation.

Bacterial culture was grown at 37^{o}C for one day, after which it was centrifuged at 5000x g, +4^{o}C for 10 minutes. The supernatant was discarded and the pellet was

dissolved in 6 ml of resuspension solution containing RNase A. The cell solution was vortexed to ensure that no cell clumps remain. Then, 6 ml of Lysis solution was added and the solution was mixed by gentle inversion for 4-6 times until it becomes a bit clear and viscous. The solution was incubated at room temperature for no more than 3 minutes. 6 ml of Neutralization solution was added to stop the reaction and the solution was immediately mixed by gentle inversion for 5-8 times. After the reaction was stopped, 0.8 ml of Endotoxin binding reagent was added and the solution was mixed again by gentle inversion for 5-8 times. The solution was incubated at room temperature for 5 minutes. Then, 96% ethanol was added and the solution was mixed by inversion for 5-6 times. Next, the solution was centrifuged at 4000-5000x g for 40 minutes to precipitate cell debris and chromosomal DNA.

The supernatant was taken to a new 50 ml falcon tube, avoiding any white residues. Later on, 6 ml of 96% ethanol was added to the supernatant and the solution was immediately mixed by inversion for 5-6 times. The sample was transferred to purification column, each time as 20 ml, and was centrifuged at 2000x g for 3 minutes. This step was repeated until all lysate was processed. Then, 8 ml of isopropanol-added Wash solution I was added to the purification column and the sample was centrifuged at 3000x g for 2 minutes.

The solution that passed through the column was discarded and the column was placed back on the tube. 8 ml of ethanol-added Wash solution II was added to the purification column, followed by centrifugation at 3000x g for 2 minutes. The flow-through was discarded and the column was placed back. The washing step with Wash solution II was repeated. After centrifugation, another centrifugation at 3000x g for 5 minutes was performed in order to remove all wash solutions from the column. The flow-through was discarded and the column was placed on a new 50 ml falcon tube. Afterwards, 1 ml of Elution buffer was added onto the center of the purification column membrane and it was incubated at room temperature for 2 minutes. Incubation was followed by centrifugation at 3000x g for 5 minutes. The last step was repeated with 0.5 ml Elution buffer in order to increase the DNA yield about 20-30%. The column was discarded after centrifugation and the concentration of purified DNA was measured using NanoDrop spectrophotometer. Aliquots of purified DNA was stored at -20^{o}C.

2.2.3 Restriction enzyme digestion and agarose gel analysis

Restriction enzyme digestion was performed in order to confirm the plasmids obtained by Maxiprep isolation. Digested plasmids were analyzed by agarose gel electrophoresis. For this purpose, restriction enzymes were selected according to the target gene. In a total colume of 20 µl reaction, 1 µl (10 units) of restriction enzyme and 2 µl of 10X CutSmart buffer was used to digest 500 µg of plasmid DNA. Remaining volume of the reaction was completed with distilled water. After mixing of the components the reaction was incubated at 37^{o}C either for 1 hour or overnight. At the end of reaction the sample was further incubated at the temperature and for the duration required to inactivate the selected restriction enzyme.

1% agarose gel was prepared by dissolving 0.6 g of agarose in 60 ml of 1X TAE buffer. The buffer was microwaved until complete dissolution occurred. The mixture was cooled down and 6 µl of Safeview dye was added before the gel was cast. After the gel solidified, samples were mixed with 6X MassRuler Loading Dye and loaded on the gel. 5 µl of MassRuler Mix marker was loaded on the gel as a band size reference. Samples were run at 90 V for 45 minutes by electrophoresis. Bands were observed under UV light.

2.2.4 Sequencing analysis

Sequencing analysis was performed to validate the plasmids obtained by Maxiprep isolation. To this aim, 30 µl of plasmid DNA samples at 100 ng/µl concentration were sent to be sequenced in a company (Macrogen). Obtained sequences were validated via comparison to the reference sequences using NCBI primer blast tool.

2.2.5 Cell culture

2.2.5.1 Thawing of HEK293T and SH-SY5Y cell lines

Cells taken from liquid nitrogen or -80^{o}C stock were immediately thawed in 37^{o}C water bath and transferred to a falcon tube containing 9 ml of pre-warmed full DMEM medium (Dulbecco's Modified Eagle Medium containing 10% FBS, 1% L-Glutamine and 1% Penicilin/Streptomycin). Cells were centrifuged at 1100 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved by tapping the falcon. Dissolved pellet was mixed with 10 ml of pre-warmed medium by gentle pipetting. Then, cells were plated on 100 mm plates and incubated at 37^{o}C

temperature and 5% CO_2 condition. On the next day, the adherence of the cells was confirmed and the medium was replaced with 10 ml of pre-warmed fresh medium.

2.2.5.2 Passage of HEK293T and SH-SY5Y cell lines

Cells were passaged to a new plate when they reached to 80% confluency. First, medium was aspirated using vacuum. Next, 5 ml of PBS was gently released from the side of the plate to wash the cells. PBS was aspirated by vacuum and 2.5 ml of the cell dissociation reagent Trypsin-EDTA (0.05%) was immediately added onto the cells to detach the adherent cells. For Trypsin enzyme to work effectively, the cells were incubated at 37^{o}C for 2 minutes. Then, 10 ml of pre-warmed full medium containing serum was added to deactivate Trypsin. Pipetting was performed to release cell clumps. Later on, cells were collected in a 15 ml falcon tube and centrifuged at 1100 rpm for 5 minutes. The supernatant was discarded and the pellet was dissolved by tapping the falcon. Resuspended cell pellet was split at 1:5 ratio and gently mixed with 10 ml medium to be plated on new 100 mm plates. Cells were further incubated at 37^{o}C and 5% CO_2.

2.2.5.3 Freezing of HEK293T and SH-SY5Y cell lines

When cells reached 80% confluency they were detached and centrifuged as described in the cell passaging protocol. Supernatant was discarded and the pellet was dissolved by tapping the tip of the falcon tube. 1 ml of freezing medium (50% DMEM, 40% FBS, 10% DMSO) per 5-7 million cells was added to the cell pellet and mixed with the cells by gentle pipetting. 1 ml of cells was transferred to each cryotube and placed in an isopropanol freezing container which was then taken to -80^{o}C freezer overnight. Cells were further stored at -80^{o}C for a maximum of 6 months. To store cells for longer durations, the cryotubes were transferred to liquid nitrogen tank.

2.2.6 Transfection

2.2.6.1 Transfection of HEK293T cells with PEI Branched reagent

PEI Branched (1 mg/ml, Sigma) was used as transfection reagent for the transfection of HEK293T cells. HEK293T cells were plated as 2x10^{6} cells per 100 mm plate 24 hours before transfection. On the day of transfection, full medium was aspirated from

plates and cells were gently washed with 5 ml of PBS. Next, 9 ml of antibiotic-free full medium was gently released from the side of the plate.

10 µg plasmid DNA, 1 ml DMEM and 30 µl PEI Branched reagent (all per 100 mm plate) were mixed in a 1.5 ml microcentrifuge tube by vortexing and incubated at room temperature for 15 minutes. 1 ml more DMEM was added to the mixture after incubation. Then, the transfection mixture was equally distributed drop by drop on the cell plate. The cells were incubated for 4 hours, after which the antibiotic-free medium was aspirated and replaced by 10 ml of pre-warmed fresh full medium. Transfected cells were further incubated for 48 hours.

2.2.6.2 Transfection of HEK293T cells with PEI Linear reagent

PEI Linear (1 mg/ml, Sigma) was used as another transfection reagent for the transfection of HEK293T cells. In this study, PEI Linear was determined to be less toxic and provided higher transfection efficiency than PEI Branched did. Thus, PEI Linear was preferred as the transfection reagent in most of the experiments.

HEK293T cells were plated as $2x10^6$ cells per 100 mm plate 24 hours before transfection. On the day of transfection, 10 µg plasmid DNA, 1 ml DMEM and 30 µl PEI Linear reagent (all per 100 mm plate) were mixed in a 1.5 ml microcentrifuge tube by vortexing and incubated at room temperature for 15 minutes. Then, the transfection mixture was equally distributed drop by drop on the cell plate. Transfected cells were incubated at 37^oC, % 5 CO_2 for 48 hours.

2.2.7 Total protein extraction

Medium was carefully aspirated from plates 48 hours after the transfection. Cells were washed by PBS twice. Then, PBS was aspirated carefully and the cell plates were placed on ice. 1 ml of NP-40 Lysis buffer at 4^oC was added to each plate and cells were incubated on ice for 30 minutes. After incubation cells were scraped from the culture dish and transferred to microcentrifuge tubes. Cells were centrifuged at 10000x g, 4^oC for 10 minutes and the supernatants containing proteins were stored as aliquots at -80^oC.

2.2.8 Nuclear protein extraction

Cells were washed with 5 ml ice-cold TBS. After the washing step cells were scraped in 500 µl TBS and collected into eppendorf tubes. Then cells were centrifuged at

1100 x rpm for 5 minutes at 4^{o}C. Supernatant was discarded and pellet was washed again in 500 µl ice-cold TBS. During the centrifugation 10 µl 100 X Protease Inhibitor Cocktail (Pi) and 1 µl DTT (1 M) were added into 1 ml NEB-A and NEB-C buffers. These buffers were stayed on ice until use. Subsequently, 400 µl NEB-A (pi and DTT added) was used to resuspend the pellet and the cells were incubated for 15 minutes at 4^{o}C before swelling and vortexed briefly. Then 25 µl (10 %) nonylphenoxypolyethoxyethanol (NP-40) was added to the mixture and centrifuged at 16000 x g for 30 seconds at 4^{o}C. Supernatant (cytosolic proteins) was collected into a eppendorf tube and stored at – 80^{o}C. Nuclear pellet was resuspend in 50 µl ice-cold NEB-C (pi and DTT added). Mixture was rocked for 15 minutes at 4^{o}C on the roller and centrifuged at 16000 x g for 5 minutes at 4^{o}C. Resulting supernatant containing nuclear proteins was transferred into eppendorf tube and aliquoted for the future use to avoid freeze-thaw. Then aliquots were stored at – 80^{o}C.

2.2.9 Measurement of protein concentration

Bicinchoninic acid (BCA) Protein Assay kit was used to measure protein concentrations of the lysates compared to a protein standard. In this calorimetric assay, bicinchoninic acid detects Cu^{+1} cations which are formed by reduction of Cu^{+2} ions in the presence of protein in alkaline medium. The reaction produces a purple color and the resulting complex exhibits strong absorbance at 562 nm.

The solvents used in protein measurement were NP-40 buffer for total proteins and NEB-C buffer for nuclear proteins. Table 2.7 describes the components of BSA protein standards used in the BCA assay.

Table 2.7: Protein standards used in the BCA assay.

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

Working reagent was prepared by diluting BCA reagent B 1:50 in BCA reagent A. 200 µl of working solution was mixed with 10 µl of a standard protein dilution or a protein sample of interest and loaded in wells of a 96 well-plate. Samples were mixed for 30 seconds and incubated at 37^{o}C for 30 minutes. At the end of incubation, protein concentrations were measured by a plate reader spectrophotometer at 562 nm absorbance.

2.2.10 SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a molecular technique used to define and separate proteins according to their electrophoretic mobility depending on their molecular weight. SDS is an anionic detergent that destroys the tertiary structure of proteins. Disulfide bonds in the proteins were destroyed by the use of dithiothreitol (DTT) in this method. SDS-PAGE was performed before western blotting experiments.

First, the components of separating gel at the gel concentration of interest according to the experiment were mixed as described in the Table 2.5. Then, the separating gel was cast in the vertical gel cassette using a pasteur pipette and the gel was left to solidify. After the separating gel has solidified, stacking gel at a concentration of interest was prepared as described in Table 2.5. The stacking gel was cast on the separating gel. Right after, a gel comb was placed to form wells on the separating gel before it was left to solidify. Cassette containing the solid gels were placed in a vertical electrophoresis chamber. The chamber was filled with running buffer and the comb was removed carefully.

Each protein sample was mixed with Sample buffer (final concentration: 1X) and DTT (final concentration: 1X) before they were loaded on the stacking gel. Protein samples were denatured at 95^{o}C on a pre-heated heating block for 5-10 minutes. Denatured proteins were then loaded on the wells of stacking gel. 5 µl of protein marker was also loaded as a reference for protein size. Electrophoresis was performed on the loaded gel at 90 V until the samples passed through the stacking gel. The voltage was set to 110 V after the samples have passed to the separating gel. The samples were run until they reached at the bottom portion of the gel.

2.2.11 Western Blot

Western blot technique helps to identify a specific protein in a given cell lysate. Blotting is performed after the proteins become separated according to their molecular weight during SDS polyacrylamide gel electrophoresis.

After SDS-PAGE was complete, the separating gel was taken apart from the stacking gel. Proteins were then transferred from the separating gel to a nitrocellulose membrane. For the transfer, Biorad's wet transfer system was used. Nitrocellulose membrane, separating gel and filter papers (Whatman) were soaked in cold transfer buffer (+4^{o}C) for 5 minutes. Transfer cassette was loaded with the materials in the following order: foam ped, filter paper (x2), gel, nitrocellulose membrane, filter paper (x2) and foam ped. The cassette was placed in the transfer tank and filled with 1X transfer buffer. The transfer system was incubated overnight at +4^{o}C and 16 V. After blotting was complete the membrane was soaked in Ponceau-S stain to check whether transfer occurred. Then, the position of the marker was noted by a pencil.

Following steps were performed after placing the membrane in a container on a shaker. The membrane was incubated in 3% non-fat dry milk in TBS-T buffer at room temperature for 1 hour to block nonspecific binding. Blocking was followed by washing of the membrane 3 times in TBS-T buffer for 5 minutes. The membrane was incubated overnight at +4^{o}C on rotator in a primary antibody solution diluted in 10% FBS in TBS-T buffer.

Next, the membrane was washed 3 times in TBS-T buffer. Then, it was incubated at room temperature for 1 hour in a suitable secondary antibody solution diluted in 3% non-fat dry milk in TBS-T buffer. The membrane was washed twice in TBS-T and once in TBS buffer each for 5 minutes at room temperature. Finally, the image of the membrane was taken using ChemiDocTM MP System (Biorad).

2.2.12 Co-immunoprecipitation (Co-IP)

2.2.12.1 α-FLAG-Co-IP and α-HA-Co-IP

Co-immunoprecipitation (Co-IP) is a molecular technique to identify a possible interaction between two proteins indirectly, where a specific antibody is used to precipitate a bait protein which potentially co-immunoprecipitates a prey protein, indicating a protein-protein interaction. In Co-IP studies, α-FLAG M2 affinity gel

(Sigma, A2220) was used to precipitate FLAG-tagged proteins and α-HA agarose (Sigma, A2095) was used to precipitate HA-tagged proteins.

20 µl of antibody-conjugated agarose resin per sample was added as 1:1 suspension to 1.5 ml microcentrifuge tube. The resin was centrifuged at 8000x g for 30 seconds and the supernatant was discarded. 1 ml of NP-40 lysis buffer was added and the resin was washed by inverting the tube, then centrifuged at 8000x g for 30 seconds. The supernatant was carefully discarded by a micropipette without disturbing the pelleted resin.Washing step with 1 ml of NP-40 was repeated twice. Next, 0.5 - 1 mg lysate of HEK293T cells was added onto the resin and incubated overnight on rotator at +4^{o}C. At the end of incubation, the sample was centrifuged at 2000x g and +4^{o}C for 2 minutes. The supernatant was taken to a new microcentrifuge tube and stored at -20^{o}C. The pellet was washed with 1 ml of NP-40 lysis buffer three times by inverting the tube. Centrifugation was performed at 2000x g and +4^{o}C for 2 minutes after each washing step and the supernatant was discarded carefully without disturbing the resin. Then, 50 µl of 1.5X Sample buffer was added to the pellet. Addition of 1 M DTT was performed at this step in α-HA-Co-IP experiments. Immunoprecipitated proteins were denatured at 95^{o}C for 5-10 minutes. Addition of 1 M DTT was performed at this step in α-FLAG-Co-IP experiments. Denatured proteins were centrifuged at maximum g at room temperature for 1 minute. The supernatant containing the immunoprecipitated proteins were taken to a new microcentrifuge tube. The precipitated proteins were either processed in SDS-PAGE immediately or stored at -20^{o}C.

2.2.12.2 Co-immunoprecipitation of endogenous proteins

Co-immunoprecipitation was performed on endogenous proteins obtained from SH-SY5Y cells. Cells were grown to confluency in 10 cm petri dishes and cellular lysates were taken from 5 plates using nuclear protein extraction protocol. For Protein G bead equilibration; a small amount of resin was transferred to a microcentrifuge tube and centrifuged at 12000 x g for 30 seconds. The supernant was discarded. The resin volume was determined by using another tube containing a known amount of dH_2O. Afterwards, the resin was washed with 1 ml NEB-C lysis buffer and centrifuged at 12000 x g for 30-40 seconds. The washing step was repeated. After washing steps NEB-C lysis buffer was added and the slurry

concentration was adjusted as 20%. The beads were stored at +4°C in lysis buffer containing 0.1% sodium azide.

For co-immunoprecipitation; 4 µg antibody was added to 1000 µg lysate. Table 2.8 shows the details for the antibody used in these experiments. The antibody and lysate mixture was incubated overnight at +4°C on an orbital shaker.

Table 2.8: Antibody used in endogenous Co-IP experiments.

Antibody	Clone	Clonality	Host	IgG Isotype	Protein A/G	Concentration
Anti-Brg1	Sc-17796	Monoclonal	Mouse	IgG_1	Protein G	1-2 µg per 100-500 µg of total protein (1 ml of cell lysate)

Afterwards, 50 µl of the 20% bead slurry (10 µl bed volume) was added to the lysate. The mixture was incubated for 1 to 3 hours at +4°C on an orbital shaker. Then, the mixture was centrifuged at 2000 x g for 2 minutes at +4°C and the supernatant was transferred to a fresh tube. The resin was washed with 1 ml ice cold NEB-C lysis buffer and centrifuged at 2000 x g for 2 minutes at +4°C. The sample was kept on ice during washing steps. The supernatant was discarded and the washing steps were repeated two more times. Then, 50 µl 1.5X sample buffer was added and the proteins were denatured by heating at 95°C for 5-10 minutes. The sample was centrifuged at maximum g for 1 minute at room temperature. The supernatant containing nuclear proteins was transferred to a fresh tube. The proteins were stored at -20°C or loaded directly into the SDS-gel.

2.2.13 In-Fusion cloning

In order to overexpress Brg1 1-344 and Brg1 342-598 domains in the cells, the inserts were cloned into the Myc-tagged pCS2+MT vector using the commercial In-Fusion HD Cloning Kit. For this, mainly the following steps were performed: amplification of the target sequence by PCR, linearization of vector by restriction enzyme digestion and In-Fusion cloning.

2.2.13.1 Design and preparation of PCR primers

In-Fusion primers for the genes Myc_Brg1, Brg1 1-344 and Brg1 342-598 were designed as described in the In-Fusion®HD Cloning Kit user manual. In-Fusion PCR

primers were designed to form a product that is homolog to the linearized vector ends. For successful insertion of the PCR fragment into the vector, 15 bases at the 5'end of the primer are required to be homolog to the 15 bases at the linearized vector end. In addition, at least 15 bases at the 3'end of the primer must include the target gene. Primer regions that are homologs with the target are called identical target sequences. Primer regions that are homologs with the vector are called non-identical target sequences. The sequences of designed forward (_F) and reverse (_R) In-Fusion primers used in this study were listed in Table 2.9.

Table 2.9: List of In-Fusion PCR primers.

Primer Name	Sequence	Tm (^{o}C)
MycBRG1_1-344_F	5' – GGCGACCTCACCATGGCATCCACTCCAG ACCCACCCCTG – 3' (39 mer)	85.2
MycBRG1_1-344_R	5' – TCACTATAGTTCTAGATCATGGCACCAT GGGCGCGGG – 3' (37 mer)	78.9
MycBRG1_342-598_F	5' – GGCGACCTCACCATGGCAGTGCCACTGC ACCAGAAGC – 3' (37 mer)	83.4
MycBRG1_342-598_R	5' – TCACTATAGTTCTAGATCAGGCAGGCGT CTGTCCTTCTGC – 3' (40 mer)	78.9
MycBRG1_F	5' – GGCGACCTCACCATGGCATCCACTCCAG ACCCACCCCTG – 3' (39 mer)	85.2
MycBRG1_R	5' – TCACTATAGTTCTAGATCAGTCTTCTTCG CTGCCACTTCC – 3' (40 mer)	76.9

In-Fusion PCR primers were purchased from Macrogen company as lyophilized. First, lyophilized primers were dissolved in distilled water at 100 µM stock concentrations. For 10 µM working concentration, 10 µl of stock primers were diluted 1:10 by adding 90 µl distilled water.

2.2.13.2 Amplification of the target sequence by PCR

Commercially available CloneAmp HiFi PCR Premix (Clontech) that can amplify DNA at high precision and efficiency was utilized in order to amplify the target DNA by PCR. Duration for the amplification was standardized using Serial Cloner software. Melting temperatures (Tm) for PCR primers was optimized by comparing

the data provided by the Serial Cloner software and Macrogen company. Table 2.10 shows the PCR conditions.

Table 2.10: Reaction conditions for the PCR with CloneAmp HiFi PCR Premix.

	Temperature (^{o}C)	Duration	Number of cycles
Initial Denaturation	98^{o}C	10 sec	-
Denaturation	98^{o}C	10 sec	5 cycles
Annealing	55^{o}C	30 sec	
Extension	72^{o}C	30 sec	
Denaturation	98^{o}C	10 sec	30 cycles
Annealing & Extension	72^{o}C	1 min	

Components of the PCR were shown in Table 2.11. The PCR reaction was prepared in two ways. The sample reaction contained the DNA template but the other reaction contained no template since it was a negative control. After the reaction was complete, the size of the PCR product was confirmed by agarose gel electrophoresis comparing with the size that was determined by the Serial Cloner software. To check the PCR product size, 10 µl of PCR product was mixed with 2 µl of 6X loading dye and loaded on 1% agarose gel. 5 µl MassRuler DNA Ladder Mix marker was also loaded on the gel for size comparison. Electrophoresis was performed at 90V for 50 minutes. Then, the band for the PCR product was viewed under UV light. After confirming the product size the reaction was repeated at a 4x volume in order to obtain a greater amount of the PCR product. The final product was purified from the agarose gel using NucleoSpin Gel and PCR Clean-Up kit as described in Section 2.2.13.7.

Table 2.11: Components of the polymerase chain reaction (PCR).

Components	Sample Volume/Reaction	Negative Control Volume/Reaction
CloneAmp HiFi PCR Premix	12.5 µl	12.5 µl
Forward Primer	5-7.5 pmol (0.2-0.3 µM)	5-7.5 pmol (0.2-0.3 µM)
Reverse Primer	5-7.5 pmol (0.2-0.3 µM)	5-7.5 pmol (0.2-0.3 µM)
Template	<100 ng	-
dH_2O	Up to 25 µl	Up to 25 µl

2.2.13.3 Linearization of vector by restriction enzyme digestion

In order to perform a successful In-Fusion the selected vector should be linearized. This was done by double digestion using two different restriction enzymes. Restriction enzymes were chosen according to the site where the insert will become

incorporated, and selected so that they digest the vector at only one region. The components of the restriction digestion reaction were shown in Table 2.12. 5 µg of vector, 5µl of buffer and 0.5 µl of each restriction enzyme was mixed. The reaction volume was completed to 50 µl with distilled water. The buffer was chosen according to the selected restriction enzymes. After the reaction was prepared it was incubated from 3 hours to overnight for the digestion. Increasing the incubation time and reaction volume enhance linearization and reduce the background. Afterwards, electrophoresis was performed to confirm the digestion. The linearized vector was purified from the agarose gel using NucleoSpin Gel and PCR Clean-Up kit as described in Section 2.2.13.7.

Table 2.12: Components of the restriction digestion reaction.

Components	Sample Volume/Reaction	Negative Control Volume/Reaction
Vector	5 µg	5 µg
Buffer	5.0 µl	5.0 µl
Enzyme	0.5 µl	-
dH_2O	Up to 50 µl	Up to 50 µl

2.2.13.4 In-Fusion cloning reaction

In-Fusion cloning reaction was performed using In-Fusion®HD Cloning Kit following the user manual. The vector was linearized as described in Section 2.2.13.3. PCR primers were designed as their 5'end was overlapping with 15 base pairs at the end of linearized vector while targeting the gene of interest. The PCR product was confirmed by electrophoresis and purified from the gel. Components of the In-Fusion cloning reaction was shown in Table 2.13.

Table 2.13: Components of the In-Fusion cloning reaction.

Components	In-Fusion Cloning Reaction	Negative Control Reaction
Purified PCR Fragment	100 ng	-
Linearized Vector	100 ng for 0.5 to 10 kb	100 ng
5X In-Fusion HD Enzyme Premix	2 µl	2 µl
dH_2O	Up to 10 µl	Up to 10 µl

100 ng of linearized vector, 100 ng of purified PCR product and 2 µl of 5X In-Fusion®HD Enzyme Premix was mixed. No PCR product was used in the negative control. The total volume was completed to 10 µl with distilled water. The reaction

was incubated at 50°C for 15 minutes and then placed on ice. The obtained In-Fusion product was used in transformation or stored at -20°C.

2.2.13.5 Construction of an expression vector by In-Fusion cloning

In-Fusion®HD Cloning Kit was used to construct a mammalian expression vector. The most critical aspect was the In-Fusion enzyme itself since it provided successful fusion of the gene of interest to the linearized vector. In addition, 15 bp overlap ends provided precise recognition and improved the fusion efficiency as the PCR amplified the target gene sequence. Along with the target, additional sequences at both ends homologous to the ends of the linearized plasmid was also amplified. In-Fusion reaction was set up right after purification and quantification of the PCR product and vector linearization. Afterwards, a portion of the In-Fusion product was transformed into competent cells. Table 2.14 outlines the general protocol to generate a new construct.

Table 2.14: Protocol to generate a new construct.

Step	Description
1	Basic vector selection and identification the insertion site. Linearize the base vector by restriction enzyme digestion.
2	Design PCR primers for 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 In-Fusion cloning reaction.
6	Transform competent cells with 2.5 µl of the reaction mix from Step 5.

In order to determine through which Brg1 domains the interaction between Brg1 and NFI proteins occurs, new expression vectors were constructed in addition to the purchased vectors pCS2+MT_Brg1_597-1407 (encodes amino acids 597-1407 of Brg1) and pCS2+MT_Brg1_1400-1648 (encodes amino acids 1400-1648 of Brg1). New expression constructs pCS2+MT_Brg1_1-344 and pCS2+MT_Brg1_342-598 were designed to encode the domains of Brg1 at the amino acids 1-344 and 342-598 respectively.

Brg1 1-344 and Brg1 342-598 domains were cloned into the pCS2+MT vector using In-Fusion®HD Cloning kit. The pCS2+MT vector purchased from Joan Massague Laboratory includes the amino acid regions 1400-1648. Through digestion of the

pCS2+MT vector by Ncol and Xbal enzymes, the vector was linearized and the gene region coding for amino acids 1400-1648 was removed at the same time.

In-Fusion primers were designed as to clone the Brg1 1-344 and 342-598 domain sites in between the digested restriction sites of the pCS2+MT empty vector. The primer pairs MycBRG1_1-344_F / MycBRG1_1-344_R and MycBRG1_342-598_F / MycBRG1_342-598_R shown in Table 2.9 were used in the PCR amplification of Brg1 1-344 and Brg1 342-598 fragments respectively. PCR conditions were optimized in Serial Cloner software as shown in Table 2.10. Brg1 1-344 and Brg1 342-598 gene fragments amplified by PCR were inserted into the linearized pCS2+MT vector using the In-Fusion®HD Cloning kit as explained in Section 2.2.13.4.

2.2.13.6 Analytic gel electrophoresis for confirmation

Agarose gel electrophoresis was performed to confirm obtained PCR products and linearized vector. For the PCR product: 10 µl of PCR product was mixed with 2 µl of 6X loading dye and loaded on 1% agarose gel together with 5 µl of the marker MassRulerMix DNA Ladder. For the linearized vector: 1 µl of sample was mixed with 0.5 µl 6X loading dye and loaded on 1% agarose gel. Both samples were run at 90V for 50 minutes and the resulting bands were visualized under UV light. The In-Fusion reaction was set according to the agarose gel analysis.

2.2.13.7 DNA purification from agarose gel

Following the confirmation of PCR products and the linearized vector as described in Section 2.2.13.6, DNA fragments were isolated from the agarose gel using commercially available NucleoSpin Gel and PCR Clean Up kit (Macherey-Nagel). DNA bands of interest were located and cut carefully under UV light, avoiding too much exposure to the UV light. The cut DNA band was placed in a microcentrifuge tube and weighed, subtracting the weight of empty tube from the total weight. For each 100 mg agarose gel with concentration less than 2%, 200 µl NTI buffer was added to the microcentrifuge tube. The sample was incubated right after for 5-10 minutes on a heat block pre-heated to 50^{o}C. The sample was vortexed every 2-3 minutes to promote rapid dissolution of the gel in the buffer. After the gel was completely dissolved the solution was loaded on the column provided in the NucleoSpin Gel and PCR Clean Up kit. Then, it was centrifuged at 11000 g for 60

seconds to remove the gel and the flow-through was discarded. This step was repeated if there was leftover gel solution that exceeded the column volume in the first loading. Afterwards, the column was washed with 700 µl of NT3 buffer and centrifuged at 11000 g for 60 seconds to remove the NT3 buffer. The flow-through was discarded and this step was repeated. Then, the column was centrifuged at 11000 g for 60 seconds without any solution to completely remove the NT3 buffer. In order to remove the possible ethanol leftover the column was incubated for 2-3 minutes on a heat block at 70^{o}C since ethanol could inhibit the effect of elution buffer. The column was then transferred to a new microcentrifuge tube and 30 µl of the elution buffer pre-heated to 70^{o}C was added. The column was incubated at 70^{o}C for 5 minutes. Next, the column was centrifuged at 11000 g for 1 minute and flow-through containing the eluted DNA fragments was obtained. The concentration of DNA fragments was determined by measuring the absorbance at A260 nm with the NanoDrop spectrophotometer.

2.2.14 Transformation

2.2.14.1 *E. coli* DH5α competent cells

E. coli DH5α competent cells were used in order to perform the transformation of the In-Fusion product mammalian expression vector. Before transformation, LB medium and LB agar plates were brought to room temperature. *E. coli* DH5α competent cells (stored at -80^{o}C) were thawed on ice. 50 µl of cells were placed in microcentrifuge tubes through gentle pipetting for each in-fusion product and negative control (empty vector). Then, 2.5 µl of In-Fusion mixture was added to each microcentrifuge tube and the samples were incubated on ice for 30 minutes. It is important to note that adding more than 5 µl In-Fusion mixture inhibits the transformation. Right after, heat shock was performed for 1 minute on a heat block pre-heated to 42^{o}C and then the mixture was incubated on ice for 5 minutes.

The following steps were performed near a bunsen burner. After the final incubation on ice, 450 µl of LB medium was added to each microcentrifuge tube and the samples were incubated at 37^{o}C on shaker for 1-1.5 hours. Then, 100 µl (1:5 dilution) was taken from the mixture by gentle pipetting and inoculated to LB agar plates containing proper antibiotics, spreading the mixture onto LB agar using a spreader. Remaining samples were centrifuged at 6000 rpm for 5 minutes and 300 µl

of the supernatant was discarded. The final 100 µl mixture (4:5 dilution) was mixed by pipetting and inoculated to LB agar plates containing proper antibiotics, spreading the mixture onto LB agar using a spreader. Inoculated LB agar plates were incubated overnight at 37^{o}C. After overnight incubation, MiniPrep isolation was performed on the chosen colonies or the plates were stored at 4^{o}C.

2.2.14.2 Stellar competent cells

Stellar compotent cells were received together with the Clontech In-Fusion®HD Cloning Kit. These cells were used in the transformation of In-Fusion reactions. First of all, stellar competent cells (stored at -80^{o}C) were thawed on ice. After thawing, 50 µl of cells were placed in microcentrifuge tubes through gentle pipetting for each in-fusion product and negative control (empty vector). Then, 2.5 µl of In-Fusion mixture was added to each microcentrifuge tube and the samples were incubated on ice for 30 minutes. It is important to note that adding more than 5 µl In-Fusion mixture inhibits the transformation. Right after, heat shock was performed for 45 seconds on a heat block pre-heated to 42^{o}C and then the mixture was incubated on ice for 1-2 minutes.

The following steps were performed near a bunsen burner. After the final incubation on ice, 450 µl of SOC medium pre-heated to 37^{o}C (provided in the Clontech In-Fusion®HD Cloning Kit) was added to each microcentrifuge tube and the samples were incubated at 37^{o}C at 225-250 rpm for 1 hour. Then, 100 µl (1:5 dilution) was taken from the mixture by gentle pipetting and inoculated to LB agar plates containing proper antibiotics, spreading the mixture onto LB agar using a spreader. Remaining samples were centrifuged at 6000 rpm for 5 minutes and 300 µl of the supernatant was discarded. The final 100 µl mixture (4:5 dilution) was mixed by pipetting and inoculated to LB agar plates containing proper antibiotics, spreading the mixture onto LB agar using a spreader. Inoculated LB agar plates were incubated overnight at 37^{o}C. After overnight incubation, MiniPrep isolation was performed on the chosen colonies or the plates were stored at 4^{o}C.

2.2.14.3 XL-10 gold ultracompetent cells

XL-10 gold ultracompetent cells produced by Stratagene® (Catalog No: 200314) are highly efficient cells for transformation. The transformation was performed according to the kit's protocol. First of all, NZY^{+} medium was brought to 42^{o}C.

Then, XL-10 gold ultracompetent cells (stored at -80^{o}C) were thawed on ice. After thawing, 45 µl of cells were placed in microcentrifuge tubes through gentle pipetting for each in-fusion product and negative control (empty vector). Then, 2 µl β-mercaptoethanol was added to each microcentrifuge tube and the tubes were gently swirled before they were incubated on ice for 10 minutes. The tubes were gently swirled every 2 minutes during incubation on ice. Afterwards, 2.5 µl in-fusion mixture containing DNA fragment and the vector was added on the cells and gently swirled. The mixture was incubated on ice for 30 minutes. Right after, heat shock was performed for 30 seconds on a heat block pre-heated to 42^{o}C and then the mixture was incubated on ice for 2 minutes.

The following steps were performed near a bunsen burner. After the final incubation on ice, 450 µl of NZY^{+} medium pre-heated to 42^{o}C was added to each microcentrifuge tube and the samples were incubated at 37^{o}C at 225-250 rpm for 1 hour. Then, 100 µl (1:5 dilution) was taken from the mixture by gentle pipetting and inoculated to LB agar plates containing proper antibiotics, spreading the mixture onto LB agar using a spreader. Remaining samples were centrifuged at 6000 rpm for 5 minutes and 300 µl of the supernatant was discarded. The final 100 µl mixture (4:5 dilution) was mixed by pipetting and inoculated to LB agar plates containing proper antibiotics, spreading the mixture onto LB agar using a spreader. Inoculated LB agar plates were incubated overnight at 37^{o}C. After overnight incubation, MiniPrep isolation was performed on the chosen colonies or the plates were stored at 4^{o}C.

3. RESULTS

BRG1 is a putative NFI interaction partner that was previously identified as such in HeLa cells by dual-immunogold labeling and co-immunoprecipitation experiments (Zhao L. et al 2005). To investigate potential interactions between BRG1 and NFI family members, we set out to perform co-immunoprecipitation assays. To this end, BRG1 and NFI were first overexpressed in HEK293T cells by appropriate expression vectors.

3.1 Analysis of BRG1 Overexpression

We overexpressed BRG1 and its interaction partner BAF170 (Xi Q et al., 2007) as a positive control by using appropriate expression plasmids. The expression plasmids pCMV5 BRG1-FLAG and pCMV5 *BAF170*-FLAG were a gift from Joan Massague (Addgene plasmid #19143 and plasmid #19142 respectively). These constructs had been prepared by the insertion of *BRG1* or *BAF170* genes into the pCMV5 vector (Xi et al., 2007).

In order to compare the transfection efficiency, HEK293T cells were transfected with plasmids expressing FLAG-tagged BRG1 or FLAG-tagged BAF170 using linear or branched form of PEI. Next, proteins were isolated from cells transfected with expression constructs and the expression of BRG1 and BAF170 proteins were detected by western blot.

In the expression analysis by western blot, we could detect BRG1 overexpression at 235 kDa (Figure 3.1, top panel). Here, linear PEI gave a higher transfection efficiency of BRG1 (Figure 3.1, lanes 1 and 6) when compared to branched PEI (Figure 3.1, lanes 2 and 7). Therefore, linear PEI was used to promote transfection efficiency in further experiments. BAF170 is predicted to have a molecular weight of 170 kDa (Wang W. et al., 1996). We could not detect BAF170 expression (Figure 3.1, top panel) and therefore could not use BAF170 as positive control in subsequent experiments.

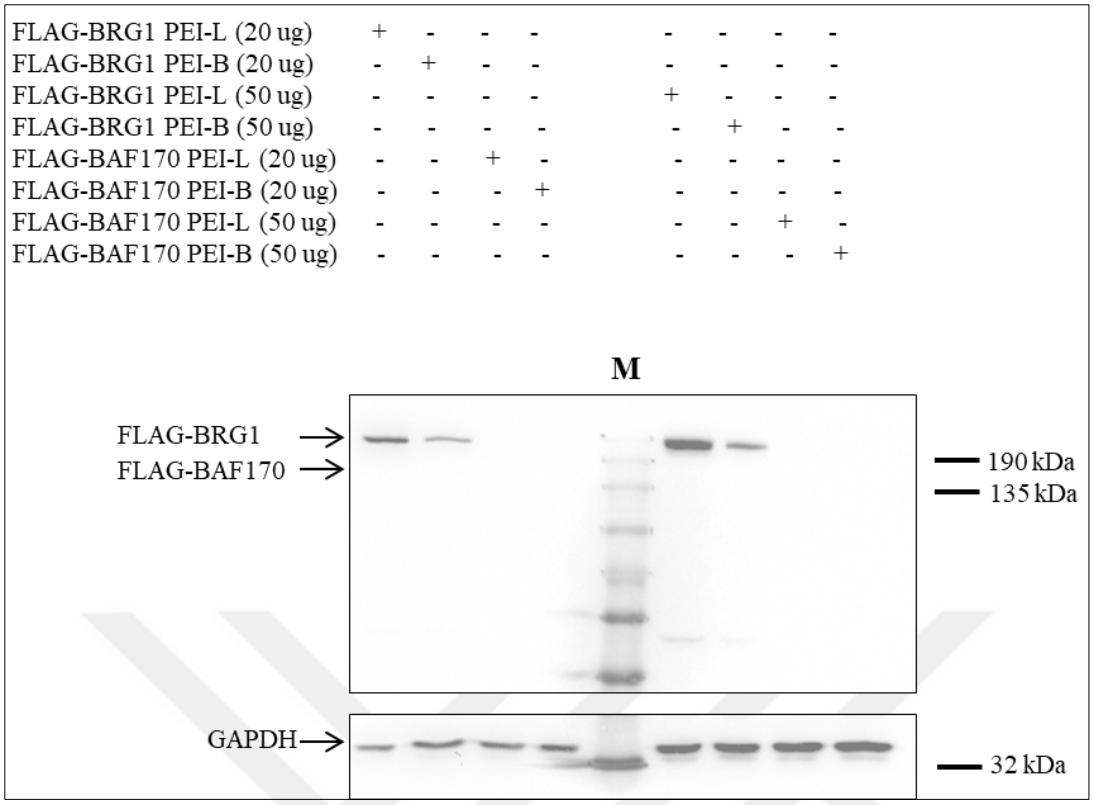

Figure 3.1: FLAG-tagged BRG1 and FLAG-tagged BAF170 expression analysis by western blot. HEK293T cells were transfected with plasmids expressing FLAG-tagged BRG1 (10 mg) or FLAG-tagged BAF170 (10 mg) using linear or branched form of PEI. Membrane extracts from transfected cells (20 µg or 50 µg) were resolved by 7.5% SDS-PAGE and detected by anti-FLAG antibody. GAPDH was included as a loading control. The position of BRG1, BAF170 and GAPDH bands are indicated by arrows. *FLAG-:* FLAG tagged, *M:* marker.

3.2 Investigation of the Interaction Between BRG1 and NFI Proteins

In order to investigate interactions between BRG1 and NFI, we first wanted to confirm that these proteins are expressed in transfected cells. HEK293T cells were transfected with plasmids that express HA-Nfib (mouse) together with FLAG-BRG1. Membrane extracts of transfected cells were subjected to Western Blot analysis using α-FLAG and α-HA antibodies.

Since BRG1 is detected at 235 kDa, co-transfected cell lysates were run on a lower concentration of 7.5% SDS-PAGE which resolves proteins with molecular weight of 46 kDa to 245 kDa. Proteins were detected by α-FLAG and α-HA antibodies. In this experiment, we detected FLAG-BRG1 at 235 kDa (Figure 3.2) and HA-Nfib at 47 kDa (Figure 3.2).

Next, co-immunoprecipitation was performed to investigate the interaction between Nfib and BRG1. Since BRG1 is a protein of high molecular weight containing many modular domains (Zhao L. et al, 2005; Xi Q. et al., 2007) and thus has potential to interact with many proteins, its interaction with ATXN3 protein which has a similar molecular weight to Nfib was analyzed as a control in co-immunoprecipitation experiments. HEK293T cells were transfected with plasmids that express FLAG-BRG1 together with HA-Nfib or HA-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 detected bands corresponding to FLAG-BRG1 at 235 kDa and HA-Nfib at 47 kDa, indicating these proteins were immunoprecipitated (Figure 3.3, lane 4). Moreover, HA-ATXN3 did not precipitate with FLAG-BRG1 (Figure 3.3, lane 5). However, we also detected additional proteins with higher mobility than BRG1 in these immune complexes. We are unsure of the identity of these additional bands (Figure 3.3, top panel).

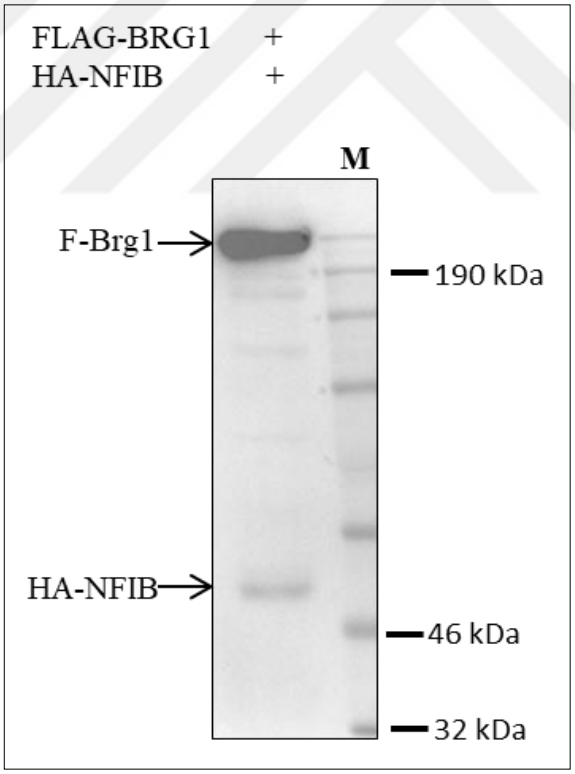

Figure 3.2: FLAG-tagged BRG1 and HA-tagged Nfib expression analysis by western blot. HEK293T cells were co-transfected with plasmids expressing FLAG-tagged BRG1 (5 mg) along with HA-tagged Nfib (5 mg). Membrane extracts from transfected cells (10 µg) were resolved by 7.5% SDS-PAGE and detected by anti-FLAG and anti-HA antibodies. The position of BRG1 and Nfib bands are indicated by arrows. *FLAG-:* FLAG tagged, *HA-:* HA tagged, *M:* marker.

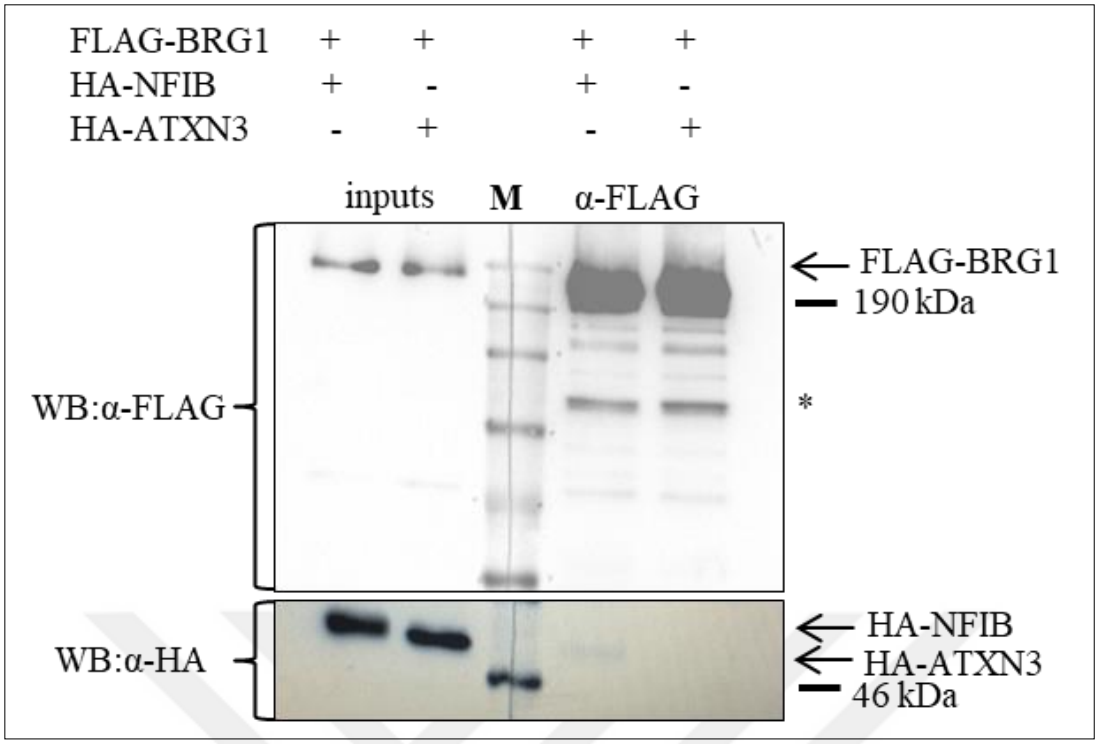

Figure 3.3: Co-immunoprecipitation analysis of FLAG-tagged BRG1 - HA-tagged Nfib interaction. HEK293T cells were co-transfected with plasmids expressing HA-tagged Nfib (5 mg) or HA-tagged ATXN3 (5 mg) along with FLAG-tagged BRG1 (5 mg). Membrane extracts were subjected to immunoprecipitation using anti-FLAG resin. Immune complexes (Lanes 4-5) or 10 µg membrane extracts (starting material, lanes 1-2) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-FLAG (top panel) or anti-HA antibodies (bottom panel). Arrows denote positions of the HA- and FLAG- immunoreactive bands corresponding to BRG1, Nfib and ATXN3. *FLAG*-: FLAG tagged, *HA*-: HA tagged, *WB:* Western blot, *M:* marker, *: Additional bands.

To determine whether Nfib protein binds to BRG1 or the FLAG resin itself during co-immunoprecipitation, empty vector (pcDNA3.1) was used as a control. HEK293T cells were transfected with plasmids that express FLAG-BRG1 together with HA-Nfib or HA-ATXN3. As control, cells were transfected with plasmids that express empty vector together with FLAG-BRG1 or HA-Nfib. Immune complexes isolated with α-FLAG resin were subjected to blotting by α-HA and α-FLAG antibodies. In this experiment, HA-Nfib co-precipitates with FLAG-BRG1 (Figure 3.4, lane 7), while HA-ATXN3, which was used as negative control, did not immunoprecipitate with FLAG-BRG1 (Figure 3.4, lane 6). Moreover, we could not detect any precipitates with the empty vector, indicating a control overexpressing FLAG-BRG1 alone (Figure 3.4, Lane 8, bottom panel). Indeed, HA-Nfib did not precipitate with anti-FLAG resin in the absence of FLAG-BRG1 (Figure 3.4, lane 9). We also

detected additional proteins with mobility higher than BRG1 in these immune complexes. We are unsure of the identity of these additional bands (Figure 3.4, top panel).

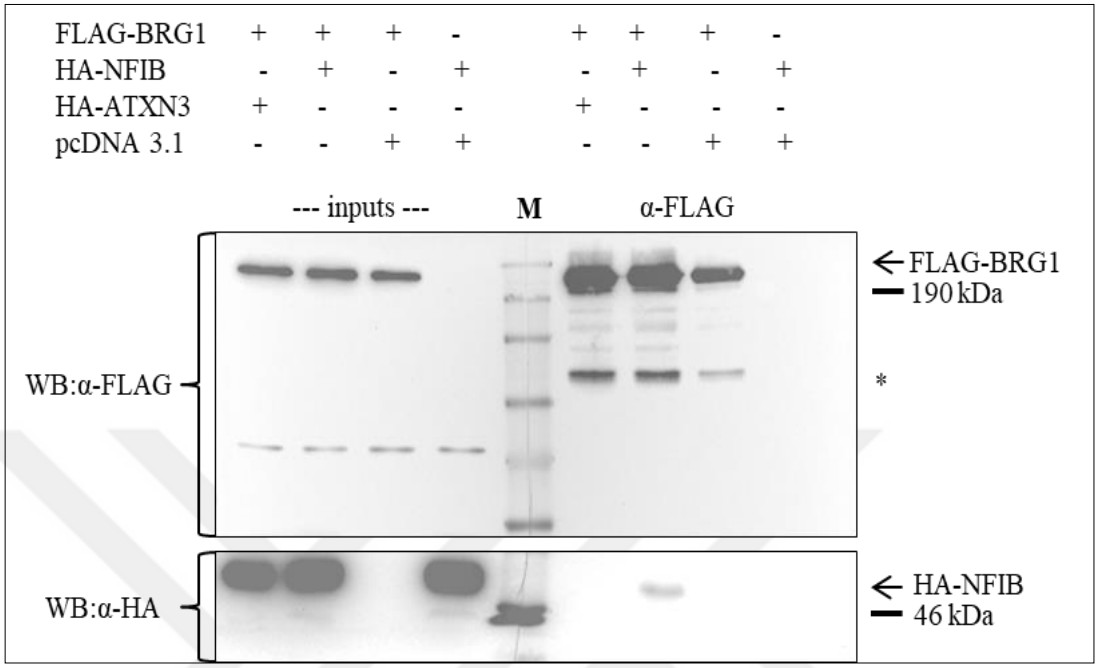

Figure 3.4: Co-immunoprecipitation analysis of FLAG-tagged BRG1 - HA-tagged Nfib interaction using empty vector (pcDNA3.1) as control. HEK293T cells were co-transfected with plasmids expressing HA-tagged Nfib (5 mg) or HA-tagged ATXN3 (5 mg) along with FLAG-tagged BRG1 (5 mg). Membrane extracts were subjected to immunoprecipitation using anti-FLAG resin. Immune complexes (Lanes 6-9) or 10 µg membrane extracts (starting material, lanes 1-4) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-FLAG (top panel) or anti-HA antibodies (bottom panel). Arrows denote positions of the HA- and FLAG-immunoreactive bands corresponding to BRG1, Nfib and ATXN3. *F-*: FLAG tagged, *HA-*: HA tagged, *WB:* Western blot, *M:* marker, *: Additional bands.

Next, we investigated whether individual NFI family members co-immunoprecipitate with BRG1. To this end, HEK293T cells were transfected with plasmids coding HA-Nfia, HA-Nfib, HA-Nfic or HA-Nfix, including negative control HA-ATXN3, along with FLAG-BRG1. Immune complexes precipitated with α-FLAG resin were subjected to blotting by α-HA and α-FLAG antibodies. In this experiment, all NFI family members precipitate with FLAG-BRG1 (Figure 3.5A, bottom panel, lanes 5 and 6) (Figure 3.5B, bottom panel, lanes 4 and 5). However, amounts of co-immunoprecipitated HA-Nfia and HA-Nfic (Figure 3.5B, bottom panel, lanes 4 and 5) are less than those of HA-Nfib and HA-Nfix (Figure 3.5A, bottom panel, lanes 5 and 6). This may be resulting from precipitation of less FLAG-BRG1 in HA-Nfia

and HA-Nfic samples (Figure 3.5B, top panel, lanes 4 and 5) when compared to those in HA-Nfib and HA-Nfix samples (Figure 3.5A, top panel, lanes 5 and 6). Also, the amount of FLAG-BRG1 in the starting material was the highest (Figure 3.5A, top panel, lane 3) in the negative control, HA-ATXN3, nevertheless HA-ATXN3 did not co-precipitate with FLAG-BRG1 as expected (Figure 3.5A, bottom panel, lane 7). However, this may be due to precipitation of least amount of FLAG-BRG1 (Figure 3.5A, top panel, lane 7) compared to other immune complexes. Then, we repeated this experiment and obtained similar results (Appendix A1-3), except this time HA-ATXN3 did co-precipitate with FLAG-BRG1 (Appendix A1, band indicated by asterisk).

We next went on to perform reciprocal immunoprecipitation experiments to test whether FLAG-BRG1 precipitates with NFIs. To this end, we used the same approach, this time immunoprecipitating HA-NFIs with α-HA conjugated agarose resin. As control, cells were transfected with plasmids that express empty vector (pcDNA3.1) together with FLAG-BRG1. In this experiment, FLAG-BRG1 precipitated in similar amounts with all NFI family members (Figure 3.6A, bottom panel, lanes 6 and 7) (Figure 3.6B, bottom panel, lanes 4 and 5). FLAG-BRG1 did not precipitate with the negative control HA-ATXN3 (Figure 3.6A, bottom panel, lane 8) although FLAG-BRG1 in the starting material was the highest among samples, along with that in HA-Nfic. Indeed, FLAG-BRG1 did not precipitate with anti-HA resin in the absence of HA-NFI members (Figure 3.6, top panel, lane 9). Then, we repeated this experiment and obtained similar results, except FLAG-BRG1 precipitated less (indicated by asterisk) with HA-Nfic compared to other isoforms although the amount of starting materials were similar (Appendix B1). Based on this assay, FLAG-BRG1 appears to interact with all NFI family members. These results are consistent with those obtained with co-immunoprecipitation experiments using α-FLAG resin (Figure 3.5).

In brief, these experiments show that HA-NFIB and HA-NFIX precipitated with FLAG-BRG1 and vice versa. Preliminary results indicate that FLAG-BRG1 can also interacts with other HA-tagged NFI members. In order to obtain conclusive results these experiments will have to be repeated, using additional HA-tagged proteins as negative control.

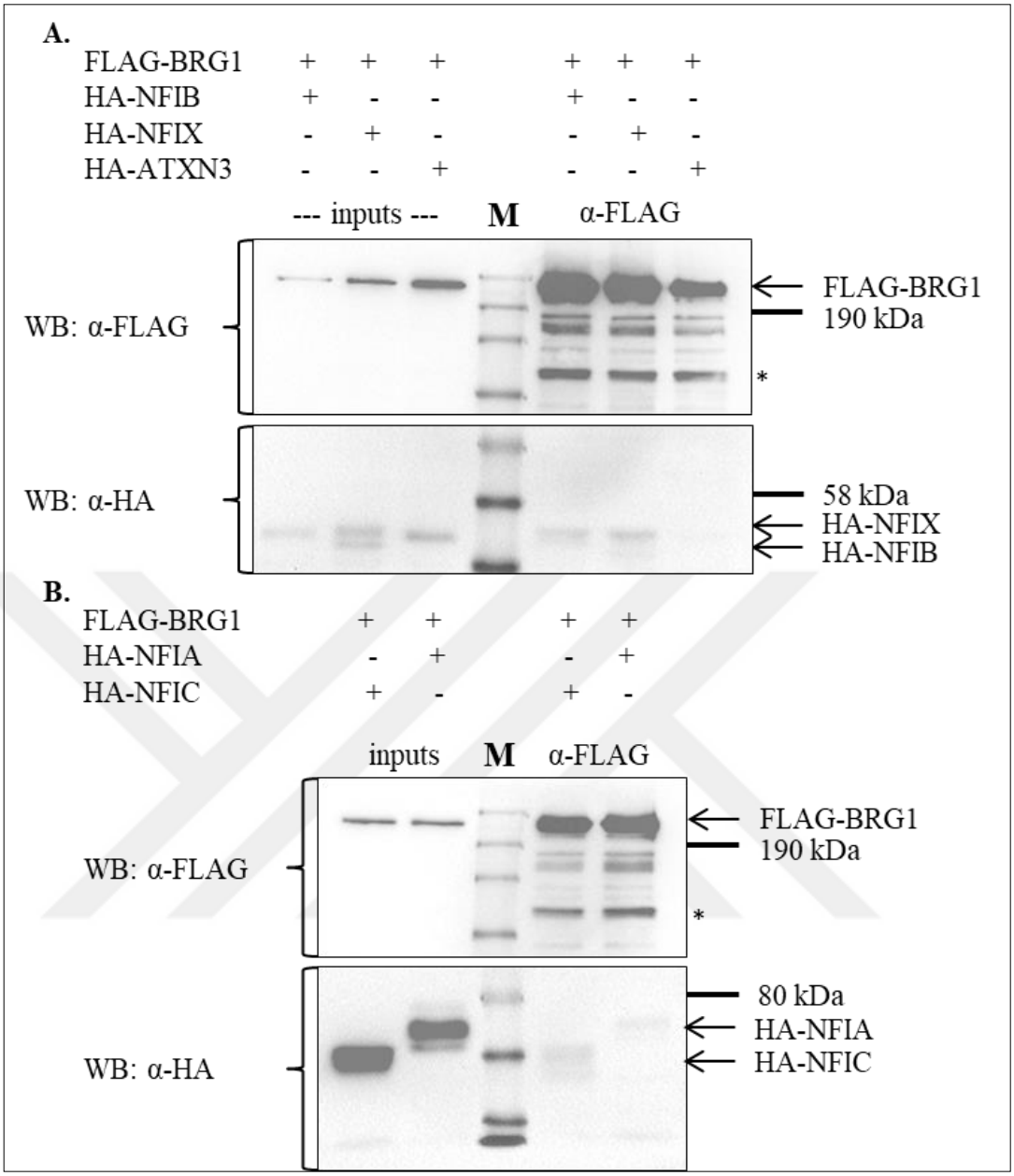

Figure 3.5: Co-immunoprecipitation by FLAG-resin to analyze the interactions between FLAG-tagged BRG1 and HA-tagged Nfi isoforms (Nfia, Nfib, Nfic or Nfix) and ATXN3. A) HEK293T cells were co-transfected with plasmids expressing HA-tagged Nfib, Nfix or ATXN3 (5 mg) along with FLAG-tagged BRG1 (5 mg). Membrane extracts were subjected to immunoprecipitation using anti-FLAG resin. Immune complexes (Lanes 5-7) or 10 µg membrane extracts (starting material, lanes 1-3) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-FLAG (top panel) or anti-HA (bottom panel) antibodies. B) HEK293T cells were co-transfected with plasmids expressing HA-tagged Nfia or Nfic (5 mg) along with FLAG-tagged BRG1 (5 mg). Membrane extracts were subjected to precipitation by anti-FLAG resin. Immune complexes (Lanes 4 and 5) or 10 µg membrane extracts (starting material, lanes 1 and 2) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-FLAG (top panel) or anti-HA (bottom panel) antibodies. Arrows denote positions of the HA- and FLAG- immunoreactive bands corresponding to BRG1, Nfia, Nfib, Nfic, Nfix and ATXN3. F-: FLAG tagged, HA-: HA tagged, WB: Western blot, M: marker, *: Additional bands.

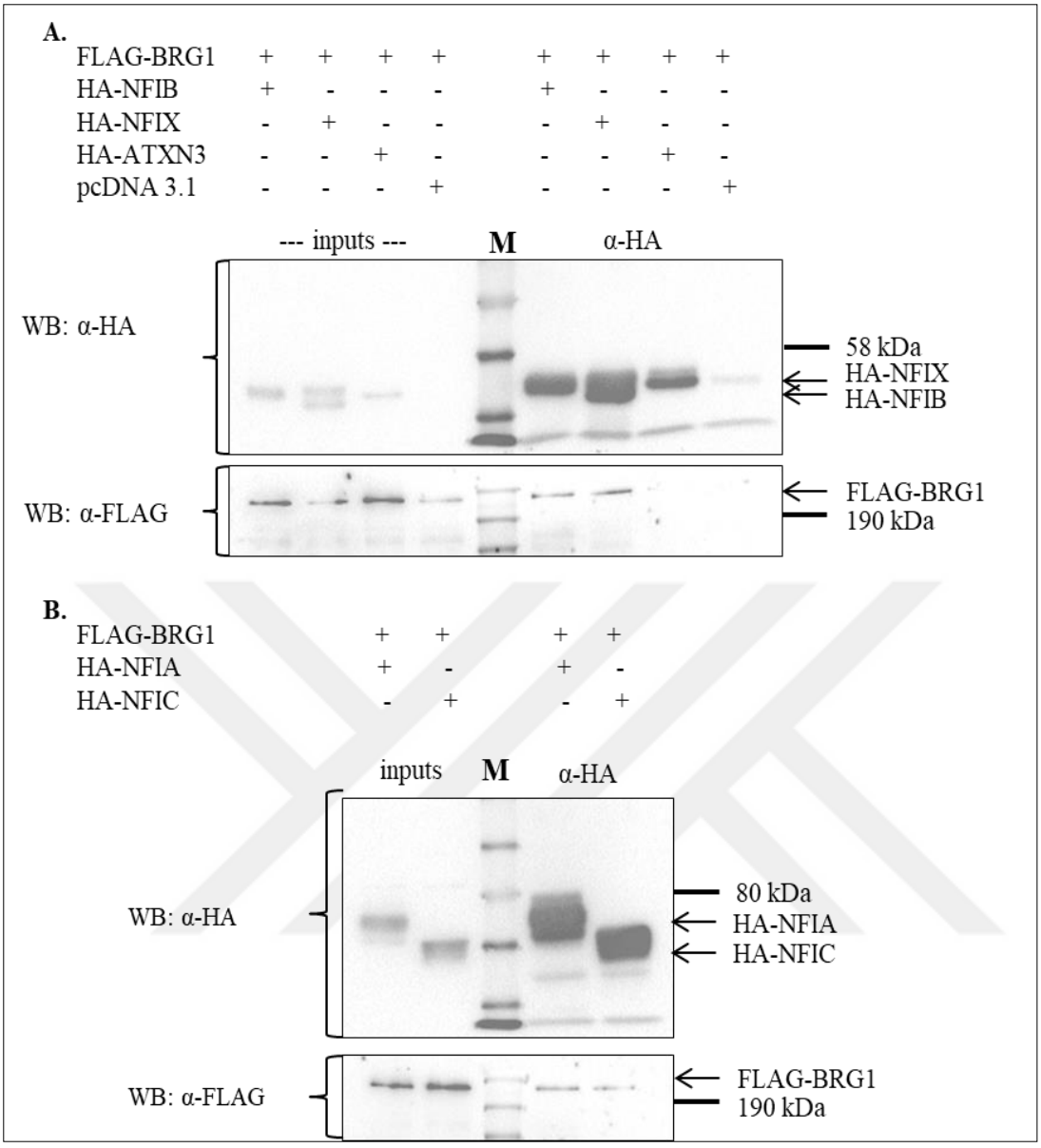

Figure 3.6: Co-immunoprecipitation by HA-resin to analyze the interactions between FLAG-tagged BRG1 and HA-tagged Nfi isoforms (Nfia, Nfib, Nfic or Nfix) and ATXN3. A) HEK293T cells were co-transfected with plasmids expressing HA-tagged Nfib, Nfix, ATXN3 (5 mg) or with empty vector (pcDNA3.1, 5 mg) along with FLAG-tagged BRG1 (5 mg). Membrane extracts were subjected to immunoprecipitation using anti-HA resin. Immune complexes (Lanes 6-9) or 10 µg membrane extracts (starting material, lanes 1-4) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-FLAG (bottom panel) or anti-HA antibodies (top panel). B) HEK293T cells were co-transfected with plasmids expressing HA-tagged Nfia or Nfic (5 mg) along with FLAG-tagged BRG1 (5 mg). Membrane extracts were subjected to precipitation by anti-HA resin. Immune complexes (Lanes 6-9) or 10 µg membrane extracts (starting material, lanes 1-4) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-FLAG (bottom panel) or anti-HA antibodies (top panel). Arrows denote positions of the HA- and FLAG-immunoreactive bands corresponding to BRG1, Nfia, Nfib, Nfic, Nfix and ATXN3. F-: FLAG tagged, HA-: HA tagged, WB: Western blot, M: marker.

Reciprocal co-immunoprecipitation experiments indicate that FLAG-BRG1 and HA-NFI proteins can interact when overexpressed in HEK293T cells. Moreover, it has been reported that BRG1 interacts with NFI in HeLa cells (Zhao L. et al, 2005) which were reported to express all NFI members (The Human Protein Atlas). However, the interactions of individual NFI family members with BRG1 were not determined by Zhao L. et al. We attempted to immunoprecipitate endogenous NFIB with BRG1 in SH-SY5Y cells because these cells express significant amounts of BRG1 and NFIB. Indeed, the amount of NFIB expression is the highest among all NFI members in SH-SY5Y cells, with 53.8 transcripts per million of NFIB compared to 51.5 of NFIX, 36.3 of NFIC and 6.6 of NFIA (The Human Protein Atlas). Nuclear extracts were prepared from SH-SY5Y to enrich for nuclear proteins and subjected to immunoprecipitation by BRG1 antibodies and Protein G beads. Precipitation by Mouse IgG antibody or Protein G Bead alone were included as control. We did not detect a higher amount of BRG1 precipitate by 8 μg BRG1 antibody compared to 4 μg (Figure 3.7, top panel, lanes 3 and 6), however, the amount of precipitated complexes with a molecular weight close to NFIB (47 kDa) increased (Figure 3.7, bottom panel, lanes 3 and 6). No precipitates were detected in the control by precipitation with Protein G beads alone. Since we also detected proteins migrating at 47 and 50 kDa in the negative control Mouse IgG (Figure 3.7, bottom panel, lane 5) we are unsure whether detected bands correspond to NFIB or Mouse IgG antibody heavy chains. However, the nuclear extracts were immunoprecipitated by mouse antibodies and then detected by rabbit secondary antibodies. Thus, in this experiment, NFIB did not precipitate specifically with endogenous BRG1. This experiment needs to be repeated with alternative negative controls.

Next, the BRG1 domain through which interaction with Nfib occurs was investigated. The structure of the human BRG1 (hBRG1) has been divided into four domains (Figure 3.8) (Xi Q. et al., 2008). Plasmid constructs expressing Myc-tagged human BRG1 domain III and domain IV, pMYC BRG1 aa597-1407 (#19146, Addgene) and pMYC BRG1 aa1400-1648 (#19145, Addgene) respectively, were obtained from Joan Massague's laboratory (Xi Q. et al., 2008). In-fusion cloning was performed in order to obtain Myc-tagged domain I, domain II and full-length BRG1 constructs. However, the constructs of interest could not be successfully cloned.

Therefore, only the interactions of Myc-tagged domains III and IV with NFIB were investigated.

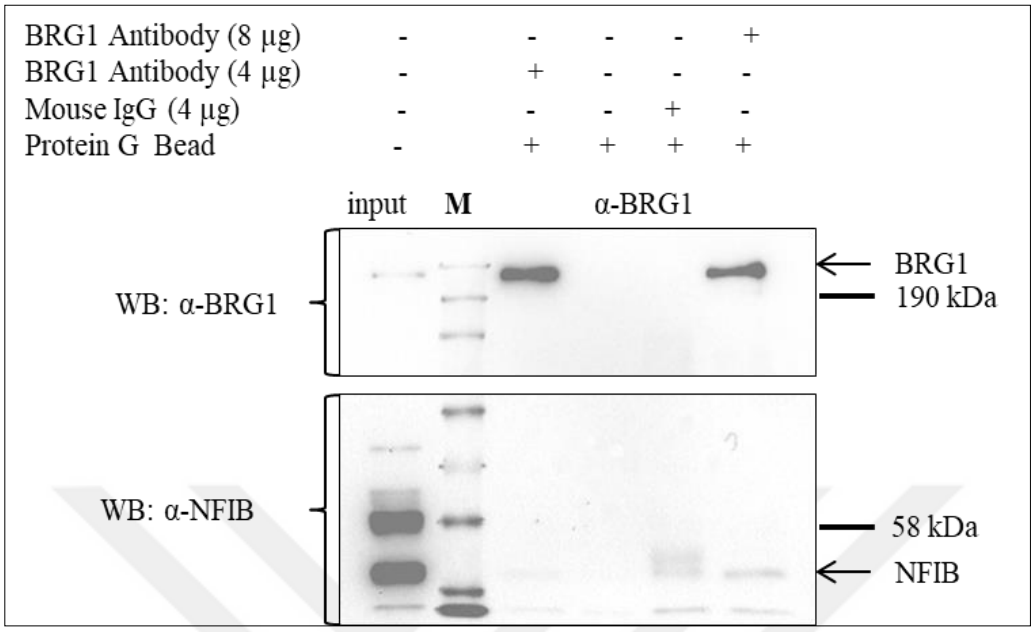

Figure 3.7: Co-immunoprecipitation of endogenous BRG1 and NFIB proteins. Nuclear extracts of SH-SY5Y cells were subjected to immunoprecipitation by anti-BRG1 antibody (4 µg or 8 µg) and Protein G beads. Immune complexes (Lanes 3-6) or 50 µg membrane extracts (starting material, lane 1) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-BRG1 and anti-NFIB antibodies. Precipitation by Mouse IgG antibody or Protein G Bead alone were included as control. Arrows denote positions of immunoreactive bands corresponding to BRG1 and NFIB. *WB:* Western blot, *M:* Marker.

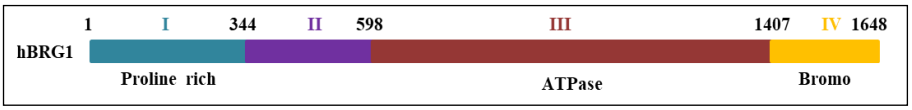

Figure 3.8: The structure of human BRG1 protein. *I:* Proline-rich domain, including amino acids 1-344, that is involved in protein-protein interactions. *II:* Conserved domain of unknown function between amino acids 342-598. *III:* Enzymatic ATPase domain involved in chromatin remodeling, between amino acids 597-1407. *IV:* Bromodomain that provides the interaction of BRG1 with acetylated histone tails, between amino acids 1400-1648.

To investigate NFIB interactions with BRG1 domains III and IV, HEK293T cells were transfected with plasmids coding FLAG-BRG1, Myc-BRG1 597-1407 or Myc-BRG1 1400-1648, along with HA-Nfib. FLAG-BRG1 was intended as positive control for precipitation. Cells were also transfected with HA-ATXN3 and empty vector (pcDNA 3.1) as control along with plasmids encoding the BRG1 domains. **I**mmune complexes were isolated with α-HA resin were subjected to blotting by α-HA, α-FLAG, α-Myc antibodies. (Figure 3.9)

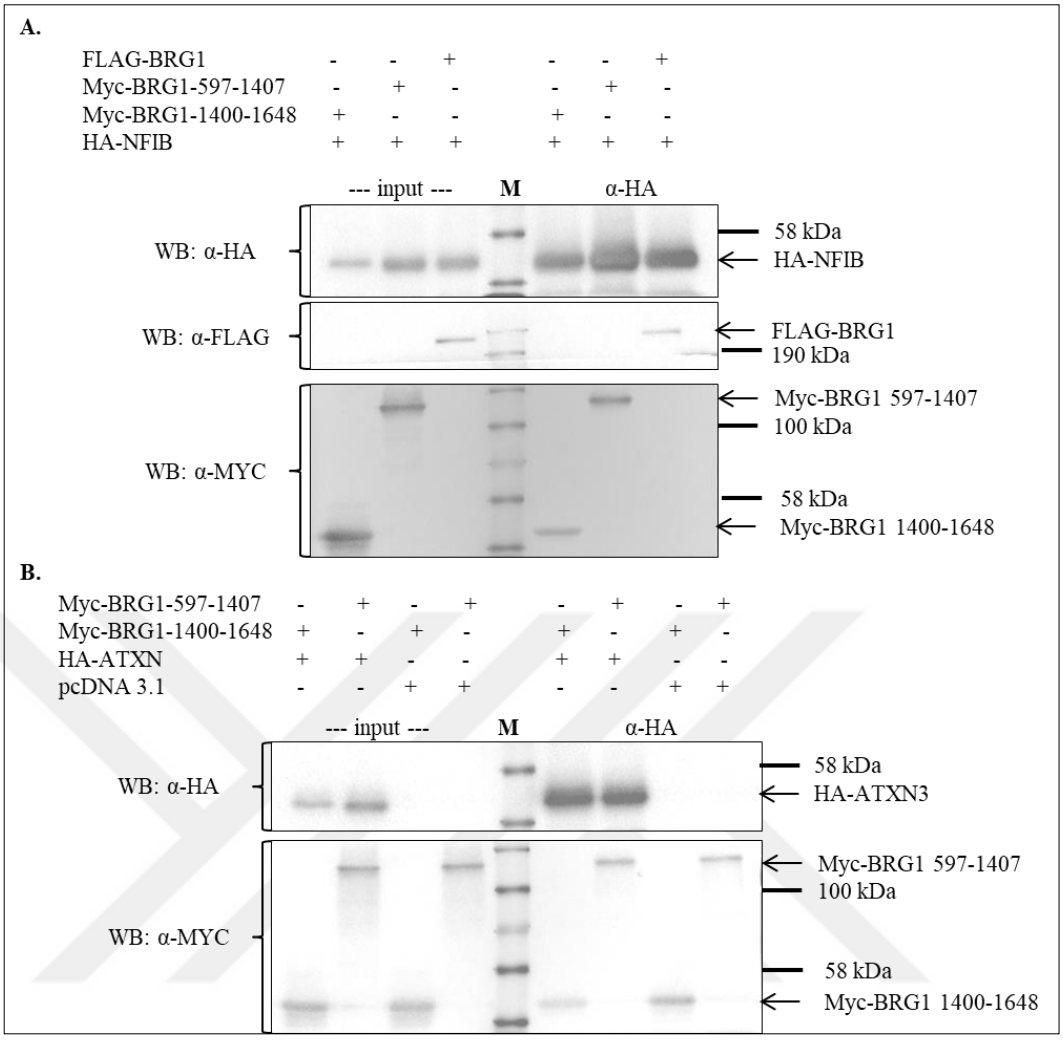

Figure 3.9: Co-immunoprecipitation by HA-resin to analyze the interactions between Myc-tagged BRG1 domains III and IV and HA-tagged and ATXN3. A) HEK293T cells were transfected with plasmids coding FLAG-BRG1, Myc-BRG1 597-1407 or Myc-BRG1 1400-1648 (5 mg), along with HA-tagged Nfib (5 mg). Membrane extracts were subjected to immunoprecipitation using anti-HA resin. Immune complexes (Lanes 5-7) or 10 μg membrane extracts (starting material, lanes 1-3) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-HA (Top panel), anti-FLAG (Middle panel) and anti-Myc (Bottom panel) antibodies. B) HEK293T cells were transfected with HA-ATXN3 or empty vector (pcDNA 3.1) (5 mg) along with plasmids coding Myc-BRG1 597-1407 or Myc-BRG1 1400-1648. Membrane extracts were subjected to immunoprecipitation using anti-HA resin. Immune complexes (Lanes 6-9) or 10 μg membrane extracts (starting material, lanes 1-4) were resolved by 7.5% SDS-PAGE followed by immunoblotting with anti-HA (Top panel) and anti-Myc (Bottom panel) antibodies. Arrows denote positions of the HA-, FLAG- and Myc- immunoreactive bands corresponding to FLAG-BRG1, BRG1 domains III and IV and ATXN3. FLAG-BRG1 was intended as positive control for precipitation. FLAG-: FLAG tagged, HA-: HA tagged, Myc-: Myc-tagged, WB: Western blot, M: marker.

While both Myc tagged BRG1 domains precipitated with HA-Nfib (Figure 3.9A, bottom panel, lanes 5 and 6), they also precipitated with the negative control HA-

ATXN3 (Figure 3.9B, bottom panel, lanes 6 and 7). Moreover, Myc-BRG1 domains also precipitated from cell lysates transfected with the empty vector (Figure 3.9, bottom panel, lanes 8 and 9) which indicate that these constructs interact with the HA-resin itself. This experiment was repeated and similar results were obtained (Appendix B2). Therefore, we are unable to ascertain whether BRG1 domains III and IV are in fact precipitated by NFIB or ATXN3. These experiments need to be carried out with alternative epitope tags or with anti-Myc resin.

4. DISCUSSION AND CONCLUSION

In this study, interactions between the enzymatic subunit BRG1 of chromatin remodeling BAF complex and NFI transcription factors were investigated. BRG1 plays essential roles in epigenetic regulation of developmental processes and cellular maintenance (Lessard J. et al., 2007; Yoo A.S. & Crabtree G.R., 2009). BRG1 protein performs ATPase activity through its DEXHc and HELICc domains, and alters the structure of chromatin to render a target gene open or close to transcription (Trotter et al., 2008). BRG1 also contains a C-terminal bromodomain which provides its binding to acetylated lysines on histones (Chandrasekaran and Thompson, 2007; Shen et al., 2007), and an N-terminal QLQ domain that permits protein-protein interactions (Kim et al., 2003; Williamson, 1994). This subunit helps changing the expression status of a gene by interacting with transcription factors within the remodeling complex.

Nuclear factor I (NFI) transcription factors bind to various DNA regions (Piper M. et al, 2014; Fletcher et al., 1999) or specific proteins (Wang W. et al., 2010; Glaskow et al., 2014) and thereby are involved in regulation of gene expression. Zhao L. et al., 2005 reported that, NFI transcription factor has been reported to interact with BRG1 in HeLa cells and activated mouse T lymphocytes. Dual-immunogold labeling studies showed that BRG1 was closely co-localized with NFI/CTF in HeLa cells. Also, co-immunoprecipitation studies have shown that NFI binds BRG1 in HeLa cells, in transcriptionally activated mouse T lymphocytes but not in resting mouse T lymphocytes (Zhao L. et al., 2005). All NFI family proteins are expressed in HeLa cells (The Human Protein Atlas) and it is unknown which NFIs are expressed in activated T lymphocytes. However, although an interaction was shown between NFI and BRG1, interactions of each individual NFI member with BRG1 were not explored. In this study, we set out to recapitulate these studies by overexpressing each NFI family member.

In order to investigate which NFI isoform BRG1 interacts with, NFIA, NFIB, NFIC or NFIX, HA-tagged NFI proteins were overexpressed in HEK293T cells along with FLAG-tagged BRG1. Extracts of transfected cells were subject to reciprocal co-immunoprecipitation assays utilizing α-FLAG and α-HA agarose resins. While FLAG-BRG1 precipitated HA-NFIB and NFIX consistently, and the other HA-NFIs inconsistently, all HA-NFI proteins precipitated FLAG-BRG1. It appears that BRG1 interacts with all NFI members *in vitro*, although further experiments need to be performed to validate these interactions.

We wanted to confirm these findings in cells that express NFI and BRG1. To this end, we prepared nuclear extracts from SH-SY5Y cells which express significant levels of NFIB, NFIX and NFIC (53.8, 51.4 and 36.3 transcripts per million respectively, The Human Protein Atlas) as well as BRG1. Immunoprecipitation of SH-SY5Y nuclear extracts with anti-BRG1 antibodies did precipitate a 47 kDa protein consistent with NFIB, however, negative control also precipitated proteins migrating at 47 and 50 kDa. Therefore, we are unable to confirm NFI-BRG1 interactions *in vivo*; these experiments need to be repeated and confirmed using alternative approaches: Reciprocal co-immunoprecipitation experiments with anti-NFIB antibody or detection by VeriBlot secondary antibody, which removes interference from denatured IgG, to overcome these additional proteins.

We also wanted to determine the BRG1 domain involved in its interactions with NFI proteins. Of the four domains of BRG1, domains III and IV (ATPase domain at amino acids 597-1407 and Bromodomain at amino acids 1400-1648 respectively) could be studied since domains I and II could not be successfully obtained by In-Fusion cloning. Myc tagged BRG1 domains III and IV were overexpressed in HEK293T cells along with HA-NFIB and nuclear extracts were subjected to immunoprecipitation with anti-HA resin. Both domains appeared to bind resin, even in the absence of NFIB. It appears that Myc-tagged BRG1 domains nonspecifically bind the HA-resin that was used for the precipitation. Therefore, we could not determine if either of these BRG1 domains binds HA-NFIB. As alternative approaches, immunoprecipitating the extracts by Myc-antibodies or *in vivo* studies such as immunofluorescence labeling can be used to investigate NFIB interactions with BRG1 domains.

In order to further investigate the functional role of the interaction between NFIB and BRG1 proteins, *Nfib* or *Brg1* knockdown experiments could be performed in neural stem cells could be established and how the knockdown of one protein affects the expression of the other protein could be examined. Furthermore, the effect of *BRG1* knockdown on the activation of NFIB target genes could be explored.

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APPENDICES

Appendix A: Repeats of FLAG-Co-IP experiments

Appendix B: Repeats of HA-Co-IP experiments

APPENDIX A

Repeats of the FLAG-Co-IP experiment were shown in Figure A.1, A.2 and A.3.

Figure A.1: First repeat of the experiment shown in Figure 3.5 in Section 3.2.

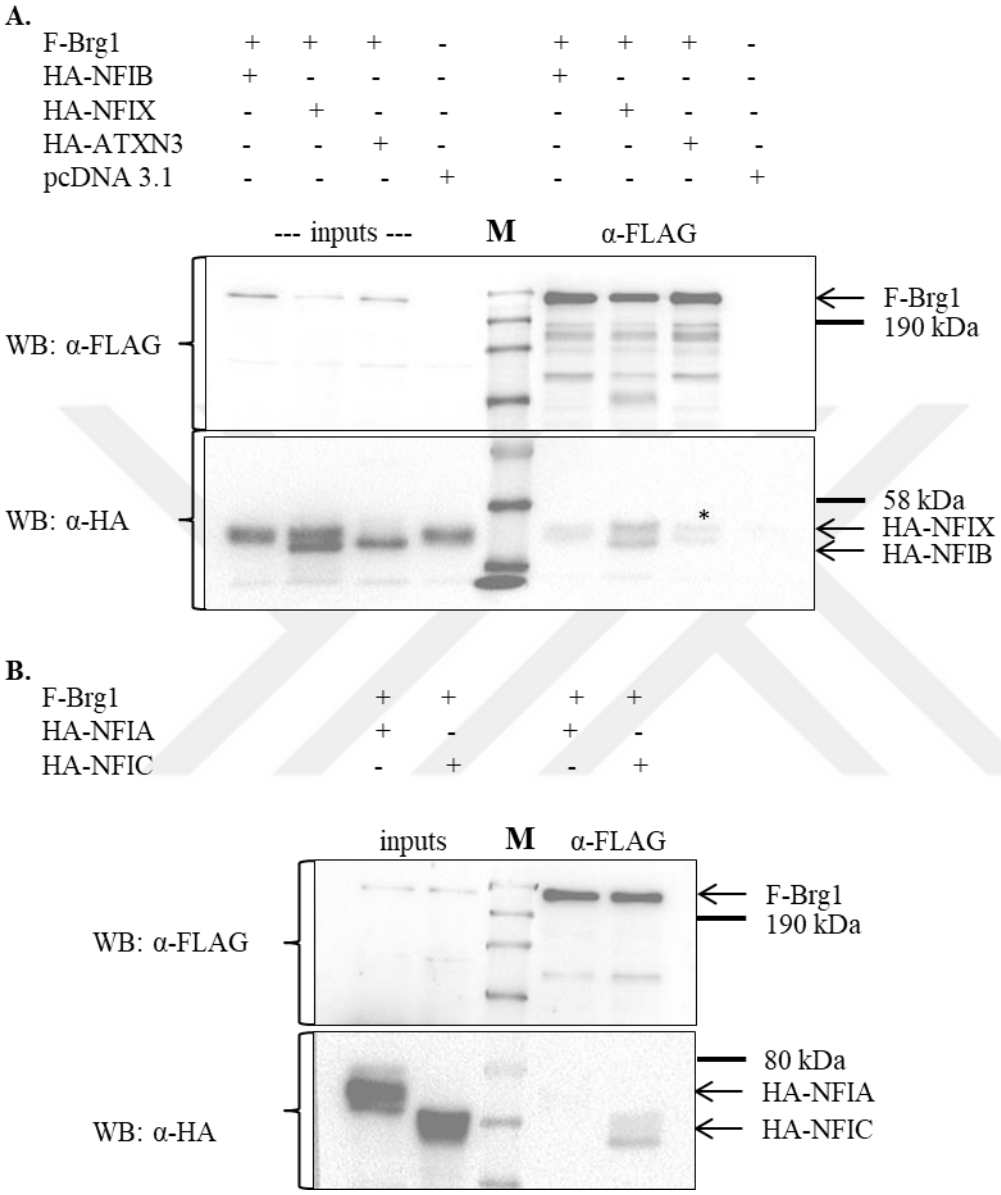


Figure A.2: Second repeat of the experiment shown in Figure 3.5 in Section 3.2.

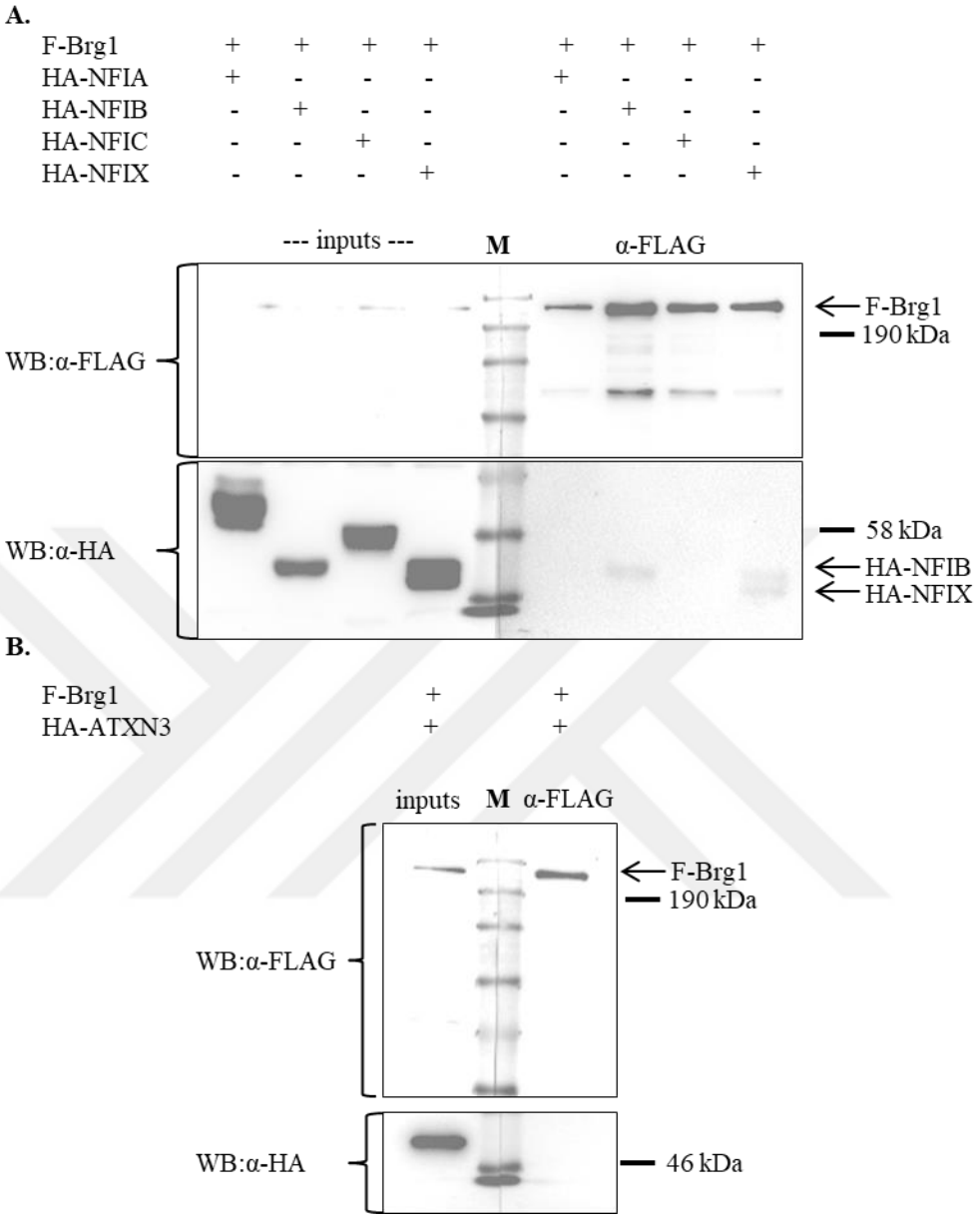
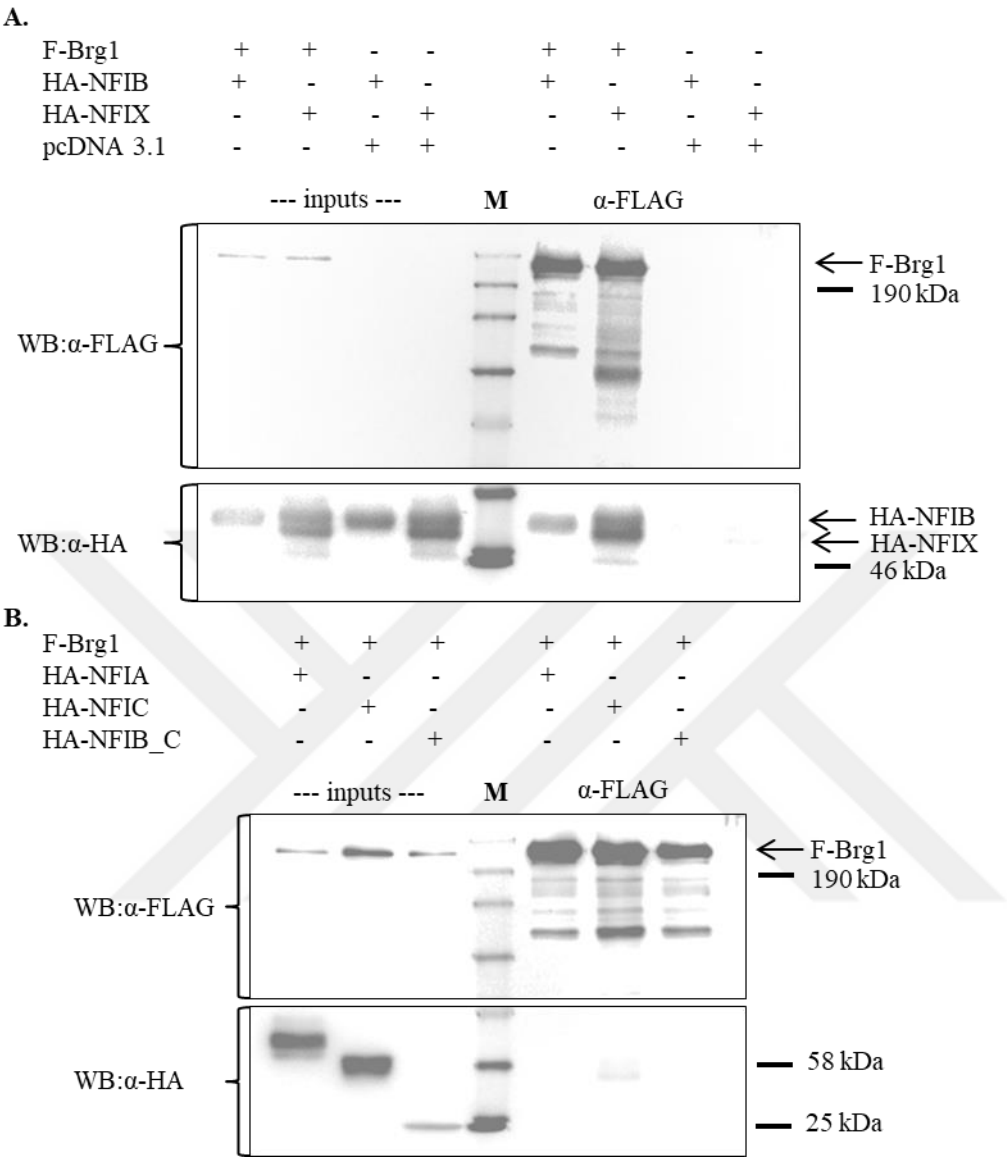


Figure A.3: Third repeat of the experiment shown in Figure 3.5 in Section 3.2.



APPENDIX B

Repeats of all HA-Co-IP experiments were shown in Figures B.1 and B.2.

Figure B.1: First repeat of the experiments shown in Figure 3.6 in Section 3.2.

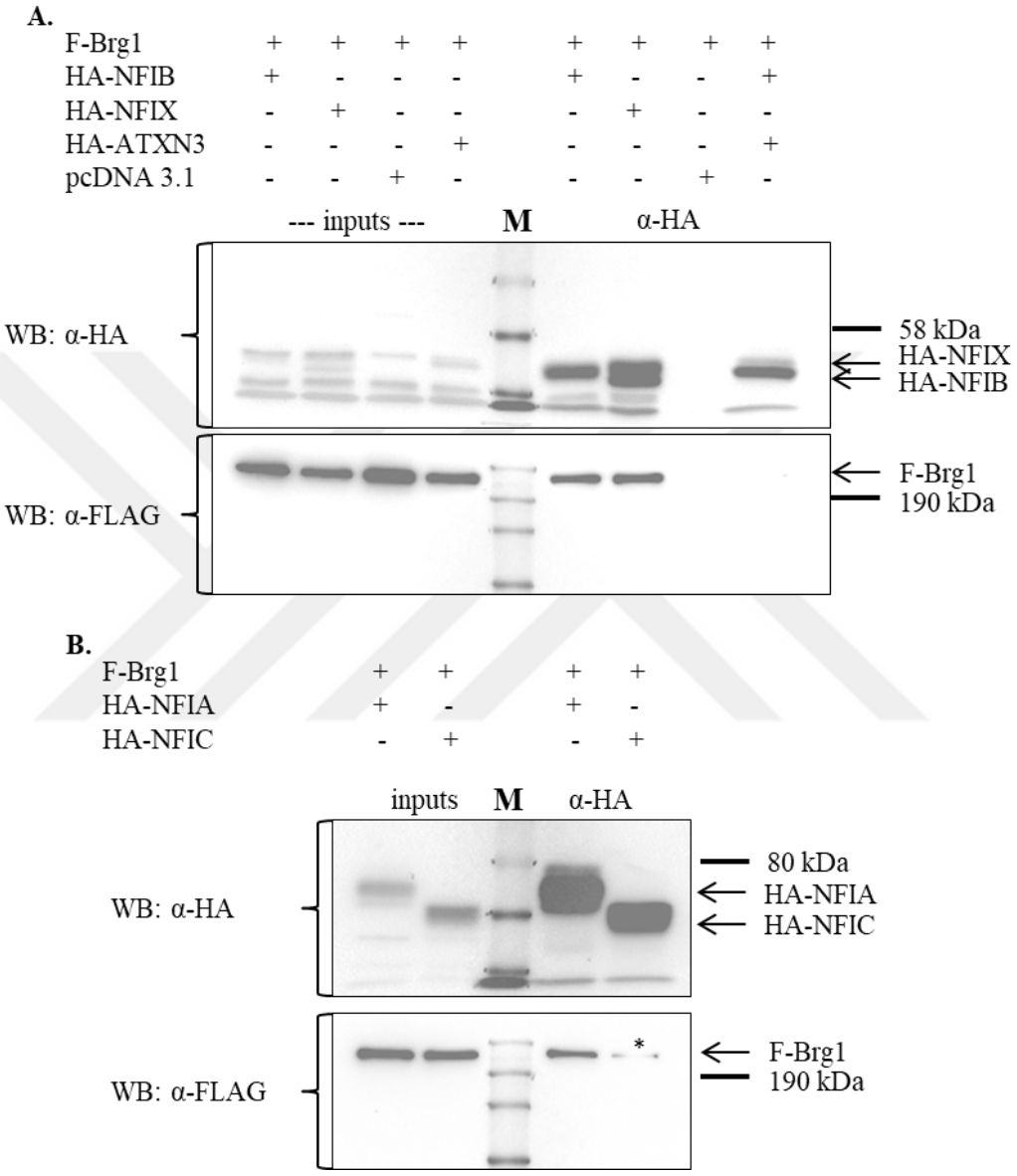
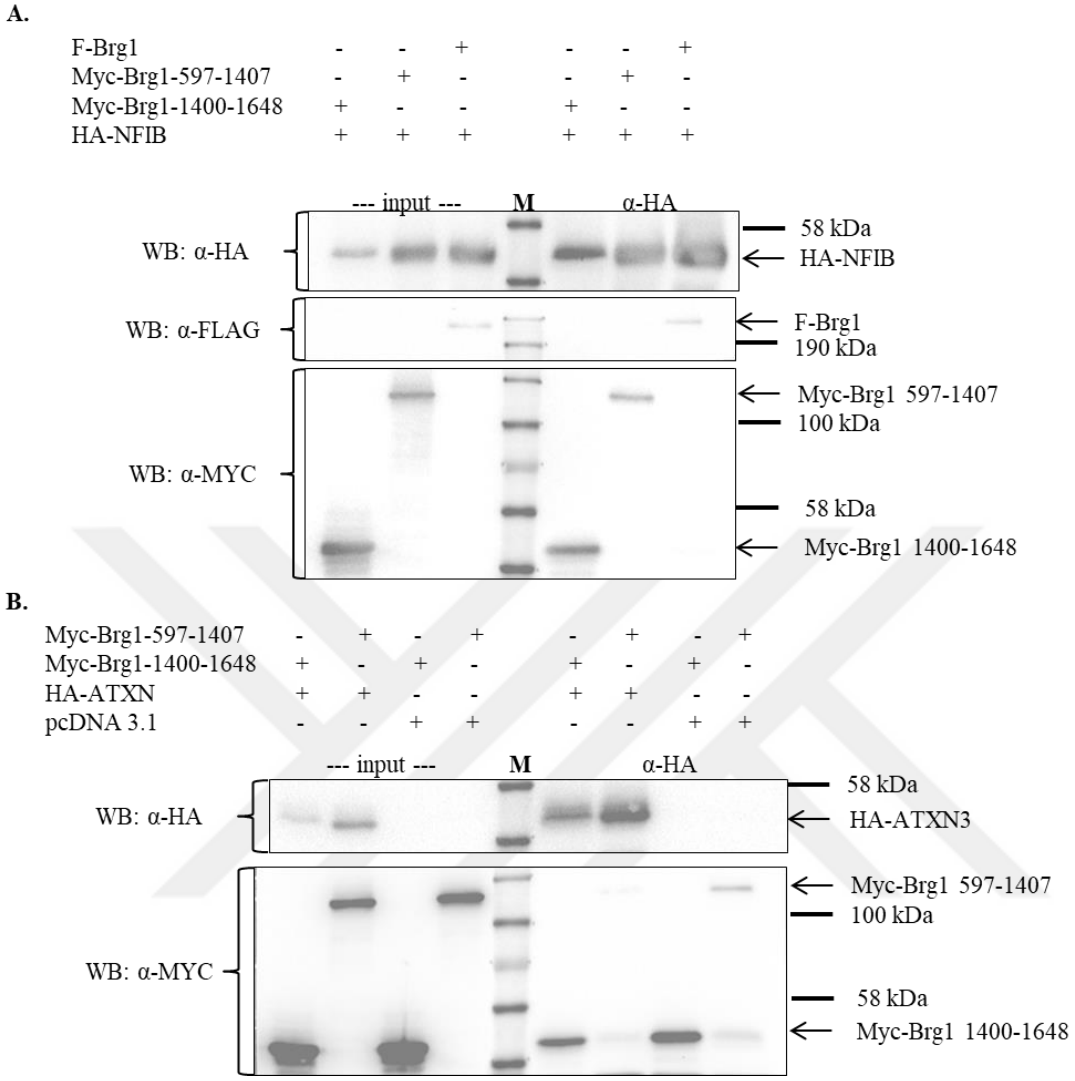


Figure B.2: Repeat of the experiments shown in Figure 3.9 in Section 3.2.



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