ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

IN VITRO INVESTIGATION OF THE INTERACTIONS OF NUCLEAR FACTOR ONE PROTEINS WITH TRANSCRIPTIONAL REGULATORS PIN1 AND LMO4

M.Sc. THESIS

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Department of Molecular Biology-Genetics and Biotechnology

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

NÜKLEER FAKTOR I PROTEİNLERİNİN TRANSKRİPSİYONEL RAGÜLATÖRLER PIN1 VE LMO4 İLE ETKİLEŞİMLERİNİN *İN VİTRO* OLARAK ARAŞTIRILMASI

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AĞUSTOS 2016

Sinem Sarıtaş, a M.Sc. student of ITU Graduate School of Science Engineering and Technology student ID 521131120, successfully defended the thesis entitled "*IN VITRO* INVESTIGATION OF THE INTERACTIONS OF NUCLEAR FACTOR ONE PROTEINS WITH TRANSCRIPTIONAL REGULATORS PIN1 AND LMO4", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

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Date of Submission : 2 May 2016
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To my family..

FOREWORD

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August 2016 Sinem SARITAŞ

TABLE OF CONTENTS

Page

ABBREVIATIONS

Amp : Ampicillin
BCA : Bicinchoninic acid
BSA : Bovine serum albumin
CNS : Central nervous system
DMEM : Dulbecco's Modified Eagle Medium
DMSO : Dimethyl sulfoxide
DNA : Deoxyribonucleic acid
dNTP : Deoxyribonucleotide triphosphate
DTT : Dithiothreitol
FBS : Fetal bovine serum
HEK : Human embryonic kidney
Kan : Kanamycin
KO : Knockout
LB : Luria-Bertani broth
MH-1 : Mad homology domain-1
MH-2 : Mad homology domain-1
NFI : Nuclear factor one
RT : Room temperature
SB : Sample buffer
SDS-PAGE : Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tm : Melting temperature

SYMBOLS

µg	**:** Microgram
µL	**:** Microliter
bp	**:** Base pair
h	**:** Hour
t	**:** Time
kDa	**:** Kilo dalton
L	**:** Liter
M	**:** Molar
mA	**:** Miliamper
min	**:** Minute
mL	**:** Density
mM	**:** Milimolar
nt	**:** Nucleotide
sec	**: S**econd
V	**:** Volt
µM	**:** Micromolar

LIST OF TABLES

Page

LIST OF FIGURES

Page

IN VITRO INVESTIGATION OF THE INTERACTIONS OF NUCLEAR FACTOR ONE PROTEINS WITH TRANSCRIPTIONAL REGULATORS PIN1 AND LMO4

SUMMARY

Nuclear factor one (NFI) family of transcription factors regulates gene expression during embryonic development and in the adult stem cell niches. NFI family comprises four members in vertebrates: NFIA, NFIB, NFIC and NFIX. NFIs bind to the consensus sequence, TTGGC(N5)GCCAA on double-stranded DNA as homodimers or heterodimers. Members of NFI family contain an N-terminal DNA binding and dimerization domain and a C-terminal transcriptional activation and repression domain. The N-terminal domain is highly conserved among members and includes a MH1 motif also found in SMAD proteins of the TGF-β signaling pathway. Proline rich C-terminal domain is less conserved and may be involved in differential protein-protein interactions between NFIs and nuclear proteins. Alternative splicing generates multiple isoforms of each member. Expression of NFIA, NFIB and NFIX is required for normal development of central nervous system, and loss of each one leads to severe defects in brain development. The fact that NFIA, NFIB and NFIX knock-out mice display distinct phenotypes suggests that each NFI has a distinct function in the brain. Interactions with different regulatory proteins may account for this specificity.

Several proteins have been found to interact with NFI family members, including the transcription factors FOXA1, Sox9, Olig2. However, proteins that bind NFI and regulate NFI function in the developing brain have not been studied well. In order to identify proteins that may interact with NFIB in the developing central nervous system, yeast two-hybrid (Y2H) screen of a human fetal brain cDNA library was previously performed. PIN1 and LMO4 were identified as putative NFIB binding partners.

PIN1 belongs to the peptidyl-prolyl isomerase family which catalyzes cis-trans isomerization of peptidyl prolyl bonds. PIN1 recognizes phosphorylated serine or threonine residues in pSer/Thr-Pro peptide sequences on target proteins. Cis-trans isomerization by PIN1 changes conformation of target proteins and affects their function. LMO4 belongs to the LIM-Only (LMO) subfamily of LIM proteins and contains two tandem LIM domains. LMO4 binds a variety of proteins via its LIM domain and acts as an adaptor that links proteins, including transcription factors, to form multiprotein complexes. In the developing brain, PIN1 and LMO4 are expressed in the right location to interact with NFIs: PIN1 is highly expressed within the ventricular zone and the subventricular zone in the cortex. LMO4 is also expressed within both ventricular zone and subventricular zone, the neurogenic regions of the cortex. Moreover, phenotypes of PIN1 and LMO4 null mice show that these proteins are involved in neural development: In the absence of PIN1 development of late-born neurons is impaired in the cerebral cortex. Absence of LMO4 leads to neural tube closure defects in the dorsal brain and defects in cortical neurogenesis affecting both early and late-born neurons.

In this study, we investigated interactions between NFIs and two putative NFIB binding partners, PIN1 and LMO4. To this end, epitope tagged PIN1 or LMO4 and NFIs were co-expressed and binding was assayed via co-immunoprecipitation experiments. We found that each NFI member and PIN1 could be co-immunoprecipitated with each other. GST pull-down assays confirmed the interactions detected in co-immunoprecipitation experiments, bacterially expressed GST-PIN1 pulled down each NFI members. To identify NFI domains required for interaction with PIN1, we created NFIB mutants that contain only the N-terminal DNA binding and dimerization or the C-terminal transactivation domains. GST pull-down experiments with these mutants showed that NFIB C-terminal domain is not involved in PIN1 interactions while the N-terminal domain is required for PIN1 binding. Site-directed mutagenesis of conserved serine residues in pSer/Thr-Pro motifs in the NFIB C-terminal domain did not interfere with NFIB-PIN1 binding, in either co-immunprecipitation or GST pull down assays consistent with the finding that NFI N-terminal domain is necessary for NFI-PIN1 interactions. On the other hand, preliminary data from co-immunoprecipitation and GST pull down experiments indicate that only NFIX interacts with LMO4. We tested the NFIB mutant that lacks the C-terminal domain for LMO4 binding via co-immunoprecipitation assays and found that it did not interact with LMO4. As NFI members differ in their ability to bind LMO4, these data suggest that the C-terminal domain of NFIX is involved in LMO4 interactions. Further site-directed mutagenesis of NFIB and NFIX can be used to pinpoint the exact regions required for PIN1 and LMO4 binding.

NÜKLEER FAKTOR I PROTEİNLERİNİN TRANSKRİPSİYONEL RAGÜLATÖRLER PIN1 VE LMO4 İLE ETKİLEŞİMLERİNİN *İN VİTRO* OLARAK ARAŞTIRILMASI

ÖZET

Nükleer faktör I transkripsiyon faktörü ailesi omurgalılarda gelişimin önemli düzenleyicisidir. Bu aile ana olarak merkezi sinir sisteminde hem projenitör hem de kök hücrelerin bulunduğu ventriküler zon olarak adlandırılan bölgede ve bunun yanı sıra akciğerler ve kas-iskelet dokusunda ifade edilmektedir. NFI ailesi NFIA, NFIB, NFIC ve NFIX olmak üzere 4 üyeden oluşmaktadır. Bu üyelerden birbirleri ile aynı veya farklı olmak üzere iki adet NFI proteini DNA çift ipliğinde TTGGC(N5)GCCAA dizisine bağlanmaktadır. NFI proteininin yapısı incelendiğinde amino (N) ucunda yaklaşık olarak 210 amino asidin oluşturduğu DNA'ya ve diğer NFI proteinlerine bağlanabileceği dimerleşme domeni, karboksi (C) ucunda ise gen ifadesini aktive ettiği veya baskıladığı C ucu domeni bulunmaktadır. Üyeler arasında amino asit dizilerinin benzerliği incelendiğinde, özellikle N-ucu domeninin üyeler arasında benzerliğinin yüksek oranda korunduğu, bunun aksine C-ucu domeninde benzerliğin daha az olduğu görülmektedir. C ucu domeninin benzerliğinin az olması, NFI proteinlerine çeşitliliği sağlamaktadır. Bunun yanı sıra, alternatif kırpılma mekanizması ile NFI üyelerinin farklı izoformlarının üretilmesi, NFI proteinlerinin çeşitliliğini arttırmaktadır.

NFI proteinlerinin diğer proteinler ile benzerliği araştırıldığında, şimdiye dek sadece SMAD proteinlerinin N ucu domeni olan MH1 domeni arasında benzerlik olduğu belirlenmiştir. Her iki protein de kısaca CCHC olarak adlandırılan sistein-sistein-histidin-sistein amino asit dizisini içermektedir. Bu korunmuş dizi her iki proteine de DNA'ya bağlanma fonksiyonu kazandırmaktadır. Bunun yanı sıra, NFI proteinleri hücre çekirdeğine girebilmek için iki adet nükleer lokalizasyon sinyali taşımaktadır. Bu sinyallerden biri NFI proteinlerini kodlayan 2.ekzonda, diğeri ise 5. ve 6. ekzonların arasında yer almaktadır.

NFI ailesi üyesi proteinlerinin fonksiyonları incelendiğinde, her birinin özel bir görev üstlenip üstlenmediği tam olarak tespit edilememiştir. Farelerde her bir NFI üyesinin ifadesinin yok edilmesi ile yapılan çalışmalarda, NFIA, NFIB, NFIC ve NFIX'in eksikliğinin merkezi sinir sisteminin ve merkezi sinir sistemi dışındaki bazı dokuların farklılaşma süreçlerini ve bu dokuların oluşumunu etkilediği görülmektedir. NFIA, NFIB ve NFIX'in ifadesinin silinmesi ile merkezi sinir sisteminde farklı fenotipler görülmektedir. Bu nedenle NFI ailesi üyesi proteinlerin birbirlerinden farklı görevlerinin olabiliceği ön görülmektedir. Üyelerin farklı regülatör proteinler ile bağlanmasının, her birine farklı görevleri kazandırabileceği düşünülmektedir.

Şimdiye kadar NFI proteinlerinin bazı proteinler ile fiziksel olarak etkileşime girdiği ve bu etkileşimin NFI'lar tarafından kontrol edilen gen ifadesinin artmasına veya azalmasına neden olduğu gösterilmiştir. Ancak beyin gelişimini etkileyen herhangi bir protein ile etkileştiği keşfedilmemiştir. Bu nedenle, daha önce insan fetal komplementer DNA (cDNA) kütüphanesinin kullanılması ile yapılan maya ikili hibrit çalışması sonucunda NFIB proteini ile fiziksel olarak etkileşime giren aday 12 adet

protein tespit edilmiştir. Bu aday proteinler arasında özellikle beyin gelişiminde önemli fonksiyonları olduğu ve çeşitli transkripsiyon faktörlerini regüle edebildiği gösterilen transkripsiyonel regülatörler PIN1 ve LMO4 proteinleri de yer almaktadır.

PIN1 ökaryotik hücreler tarafından ifade edilen, hedef proteinlerde bulunan ve prolin ile devam eden serin/treonin (S/T-P) rezidülerini tanıyarak sis-trans izomerizasyonunu gerçekleştiren bir peptidil-prolil izomerazdır. İzomerizasyon sonucunda hedef proteinlerin konformasyonunu değiştirerek bu proteinlerin yeni proteinlerle etkileşmesini sağlayabilmekte, bu proteinleri hücresel mekanizmalar tarafından yıkılmaktan koruyabilmekte veya tam aksine yıkılmalarını sağlayabilmektedir. PIN1 proteininin etkileştiği pek çok transkripsiyon faktörü ve diğer proteinler tespit edilmiştir. Bu etkileşimler sonucunda PIN1 proteininin hücrelerin bölünme veya farklılaşması süreçlerinde etkin rol oynadığı bilinmektedir. Transkripsiyon faktörleri ile olan etkileşimi sonucunda, bu faktörler tarafından kontrol edilen gen ifadesini arttırdığı veya azalttığı gösterilmiştir.

LMO4 ökaryotik hücreler tarafından ifade edilen, hücre içerisinde transkripsiyon faktörleri de dahil olmak üzere pek çok proteine bağlanabilme kapasitesine sahip bir adaptör proteindir. LMO4, LIM protein ailesine dahil olan ve LIM Only olarak adlandırılan alt aileye aittir. LIM protein ailesine üye olan proteinler, en az bir adet, aileye adını veren LIM domeni içermektedir. LMO4 proteini 2 adet birbiri ardına gelen LIM domeni içermekte, bu iki domen kısa bir bağlayıcı bölge ile birbirine bağlanmaktadır. LIM domeninin amino asit sekansı incelendiğinde, birbirlerine göre konumları değişmeyecek şekilde ard arda iki adet sistein-sistein-histidin-sistein amino asid dizisinin var olduğu görülmektedir. Her bir LIM domeni iki adet birbirini takip eden ilmek oluşturmaktadır. Çinko parmak olarak adlandırılan bu motif, hücre içerisinde çeşitli proteinler ile etkileşmek için önemli bir yüz oluşturmaktadır. LMO4 proteinlerinin DNA'ya bağlanabildiği henüz gösterilmemiştir. Ancak etkileşime girdiği proteinler aracılığı ile DNA'ya çağırılan LMO4'ün, etkileşime girdiği transkripsiyon faktörlerinin fonksiyonunu regüle edebildiği gösterilmiştir.

PIN1 ve LMO4 embryonik gelişim sürecinde ventiküler zonda ifade edilmektedir. Aynı bölgede NFI ifadesinin de var olması, ayrıca her iki aday proteinin de nöronal gelişimde önemli rollerinin olması, eksikliklerinde ise hem nöronal gelişimin hem de belirli dokuların oluşumunda bozuklukarın olması, seçilen aday proteinlerin NFI üyelerinin de işlevini regüle edebileceğini göstermektedir. Her iki aday protein de NFI proteinlerinin benzerlik taşıdığı tespit edilen SMAD ailesi üyeleri ile etkileşime girmekte ve SMAD'lerin işlevini regüle etmektedir. Bu durum, her iki aday proteinin de NFI proteinleri ile de bağlanabileceği göstermektedir.

Bu çalışmanın amacı, daha önce maya ikili hibrit deneyleri sonucunda NFIB proteini ile etkileşime girebileceği ön görülen aday proteinler PIN1 ve LMO4'ün NFI ailesine ait proteinler arasındaki etkileşimin biyokimyasal yöntemler ile gösterilmesidir. Bu amaçla ilgilenilen proteinler öncelikle N ucu domenlerinde uygun epitop peptidlerini de içerecek şekilde memeli hücrelerinde ifade edilebilecekleri ifade vektörlerine kopyalanmıştır. Ardından NFI ailesi üyelerinin PIN1 veya LMO4 proteinlerinden biri ile HEK293T hücrelerinde birlikte ifade etmeleri sağlanmıştır. Hedef proteinlerin, hücrelerin proteinleri denatüre etmeyecek bir metot ile parçalanmasının ardından elde edilen hücre lizatından, üretildikleri epitop peptidlerine uygun olarak kullanılan antikorlar aracılığıyla çökmeleri sağlanmıştır. Yapılan analiz ile hedef protein ile birlikte, aday etkileşim proteinin çökmesi incelenmiştir. Buna alternatif olarak, yine HEK293T hücrelerinde ifade edilen NFI proteinler ile *E.coli* BL21 suşu bakteri

hücrelerinde GST proteini ile füzyon proteini olarak ifade etmeleri sağlanmış olan PIN1 ve LMO4 proteinleri arasındaki etkileşim, GST'nin çöktürülmesi ile NFI proteinlerinin de bu proteinler ile birlikte çökmesi araştırılmıştır. Elde edilen sonuçlar PIN1 ile tüm NFI ailesi üyelerinin bağlanabileceğini desteklemektedir. Domen analizi ve mutasyon çalışmaları, tespit edilen bağlanmanın sağlanmasında NFI'ların N-ucu domeninin etkileşim için gerekli olabileceğini işaret etmektedir. Bunun haricinde NFI ailesi ile etkileşim için proteinlerin fosforilasyon durumlarının önemli olup olmadığı incelenmesi amacıyla fosfataz uygulaması yapılmıştır. Elde edilen veriler proteiner arasındaki etkileşimin fosforilasyona bağlı olmadığını düşündürse de, uygun bir pozitif kontrolün bulunmaması nedeniyle sonuçlar kesinlik kazanamamıştır. LMO4 ve NFI proteinleri arasındaki etkileşim aynı metotlar ile incelendiğinde, bulgular LMO4'ün NFI ailesinden NFIX ile etkileşime girebildiğini göstermektedir. NFIB proteininin N ucu domeni ile LMO4 arasında etkişim tespit edilememiştir. Bu nedenle NFIX'in C ucu domeninin LMO4 ile etkileşim için gerekli olduğu tahmin edilmektedir. NFIB ve NFIX proteinlerinde yapılacak diğer mutasyonlar ile, sırasıyla PIN1 ve LMO4 ile bağlanabilmeleri için gerekli olan domen veya rezidülerin bulunması planlanmaktadır.

Sonuç olarak LMO4 ve PIN1, NFI ailesi üyesi veya üyeleri ile etkileşime girmektedir. Bu etkileşimin NFI işlevini kontrol edip etmediği henüz bilinmemektedir. İleriki çalışmalarda LMO4 ve PIN1'in NFI ailesi üyeleri ile bağlanmasının NFI'ın gerek hedef DNA dizisine bağlanmasını, gerekse gen ifadesi üzerindeki etkisini değiştirip değiştirmediği ve eğer değiştiriyorsa, bu etkinin hücrelerde işlevsel olup olmadığı incelenmelidir.

1. INTRODUCTION

1.1 Nuclear Factor One (NFI)

Nuclear factor one (NFI) family of transcription factors are important regulators of development, both in the vertebrate embryo and in the adult stem niches. NFI family is mainly expressed within the central nervous system (CNS), found in the ventricular zones populated by stem cells and progenitors, as well as the lung and skeletal-muscles. There are four members of NFI family in vertebrates: NFIA, NFIB, NFIC and NFIX. Each NFI family member contains an N-terminal DNA binding and dimerization domain and a C-terminal transcriptional activation and repression domain (Figure 1.1). The N-terminal DNA binding domain, constituted by approximately the first 210 amino acids, is highly conserved between family members (Figure 1.2, Figure 1.3). This domain is responsible for high-affinity binding to the consensus sequence, TTGGC(N5)GCCAA on double-stranded DNA (Mason et al., 2008). NFIs are able to form both homo- and heterodimers. However, dimer formation depends on co-translation of proteins; they do not assemble as dimers if they are translated separately (Sippel and Kruse, 1994). Additionally, the N-terminal domain includes a conserved Cys-His motif required for binding to DNA. The proline rich C-terminal domain is less conserved compared to the N-terminal domain (Figure 1.2), and is involved in activation or repression of transcription of target genes (Mason et al., 2008). Two Nuclear Localization Signals (NLSs) have been identified in all NFI members (Imagawaa et al., 2000). Lysine and arginine rich residues in exon 2 and exon 5/6 are required for complete translocation of NFIs into nucleus (Figure 1.3) (Imagawaa et al., 2000). An isoform that lacks the exon which contains one of the NLSs is not completely translocated, suggesting that hetero-dimerization with this NFI can inhibit translocation of other NFIs that carry both NLSs (Imagawaa et al., 2000). Diversity of C-terminal domains confers uniqueness to each NFI family member and allows them to interact with other transcriptional regulators to control specific processes.

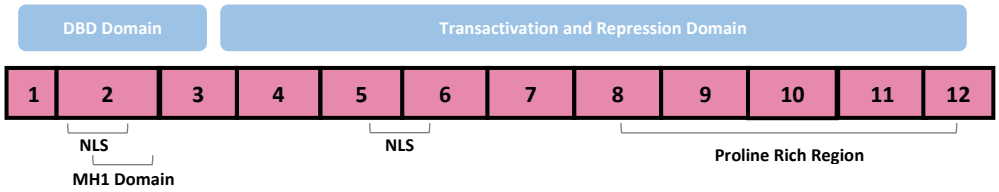

Figure 1.1 : Domain structure of NFIs. Numbers from 1 to 12 represent exons coding NFI. N-terminal region contains DNA Binding and Dimerization Domain (DBD Domain). C-terminal region contains transactivation and repression domain. Nuclear localization signals (NLSs) are found both in exon 2 and exon5/6. Mad homology domain 1 (MH1) is found in exon 2 and provides

To date, the only homology that was found between NFIs and other transcription factors is the MH1 domain located in the N-terminal region that resembles the MH1 domain of Smad proteins (Sadreyev and Grishin, 2003). Smads are transcriptional regulators involved in TGF-β signaling pathway, and contain N-terminal MH1 and C-terminal MH2 domains (Miyazono, 2000). The N-terminal MH1 domain of Smads includes two subdomains: one of these is responsible for binding to the major groove of DNA while the C-terminal MH2 domain mediates protein-protein interaction (Sadreyev and Grishin, 2003; Miyazono, 2000). Four conserved residues, three cysteines and one histidine make up a metal binding Cys-His box in the Smad MH1 domain. Interestingly, this conserved Cys-Cys-His-Cys motif at the N-terminal domain of NFIs is significantly similar to the zinc binding Cys-His box of MH1 domain of Smads (Sadreyev and Grishin, 2003). MH1 is homologous to His-Me homing endonucleases, and NFIs may have evolved as modified endonucleases which have lost their enzymatic activity but are still able to bind DNA (Sadreyev and Grishin, 2003; Grishin, 2001).

It is not known whether each member of the NFI family has a unique function. While NFIs bind to the same DNA sequence due to their highly conserved DNA binding domain, divergent C-terminal domains of each member might allow each to have specific interactions and roles (Figure 1.2, Figure 1.3). Knockout (KO) studies show that expression of each member alone is required for normal development of tissues regulated by these NFI members, and loss of each one results in severe developmental defects (Piper et al., 2010; Steele-Perkins et al., 2005; Piper et al., 2014; Heng et al., 2014, Messina et al., 2010). NFIA, NFIB and NFIX KO mice show delayed neuronal and glial differentiation and impaired brain development (Piper et al., 2010; Steele-Perkins et al., 2005; Heng et al., 2014). NFIC KO mice do not exhibit a phenotype

associated with central nervous system possibly due to the low expression level of NFIC within the CNS. In addition, NFIB, NFIC and NFIX KO mice show impaired development of lung, tooth root and muscle-skeletal tissue, respectively (Piper et al., 2010; Steele-Perkins et al., 2005; Piper et al., 2014; Heng et al., 2014, Messina et al., 2010). The fact that knockout mice where each NFI is deleted indvidually display similar phenotypes in the CNS suggests that NFIs may have similar functions. Additionally, deletion of one member results in increased expression of other member(s) suggesting that loss of any NFI might be compensated by increased expression of other NFIs and the overall expression level may be more important than function of individual NFIs (Harris et al., 2015; Mason et al., 2008).

In addition to four members of NFI encoded by different genes, multiple isoforms are produced for each member by alternative splicing. NFIA has at least four, and the other members have at least six isoforms in humans (available from UniprotKB with ID Q12857, O00712, P08651, Q14938). Except for the short isoform of NFIB, all splice variants maintain the canonical protein structure which includes the N-terminal DNA binding domain and C-terminal transacivation/repression domain. This novel NFIB isoform has been identified recently and is unable to bind DNA and dimerize with other members of NFI family since it comprises only the C-terminal domain and lacks the N-terminal DNA binding and dimerization domain (Chen et al., 2014). This NFIB isoform plays important roles in megakaryocyte differentiation (Chen et al., 2014).

	NFIA & NFIB	NFIA & NFIC	NFIA & NFIX	NFIB & NFIC	NFIB & NFIX	NFIC & NFIX
Full length	61.6	66.7	70.7	64.1	60.3	60.7
N terminal	86.9	85.9	88.6	81.2	84.8	81.9
C terminal	45.5	52.1	54.2	49.8	41.7	42.7

Percent homology

Figure 1.2 : Homologies of NFI family members. Homologies of NFIs in mouse were calculated using sequence alignment program LALIGN of Biology WorkBench 3.2 software. The first 210 amino acids were used for calculation of homology of N-terminus, and remaining amino acids were used for calculation of homology of C-terminus of NFIs. GI numbers of NFIA, NFIB, NFIC and NFIX used are GI:1524153, GI:15679966, GI:1524163, GI:13277741, respectively.

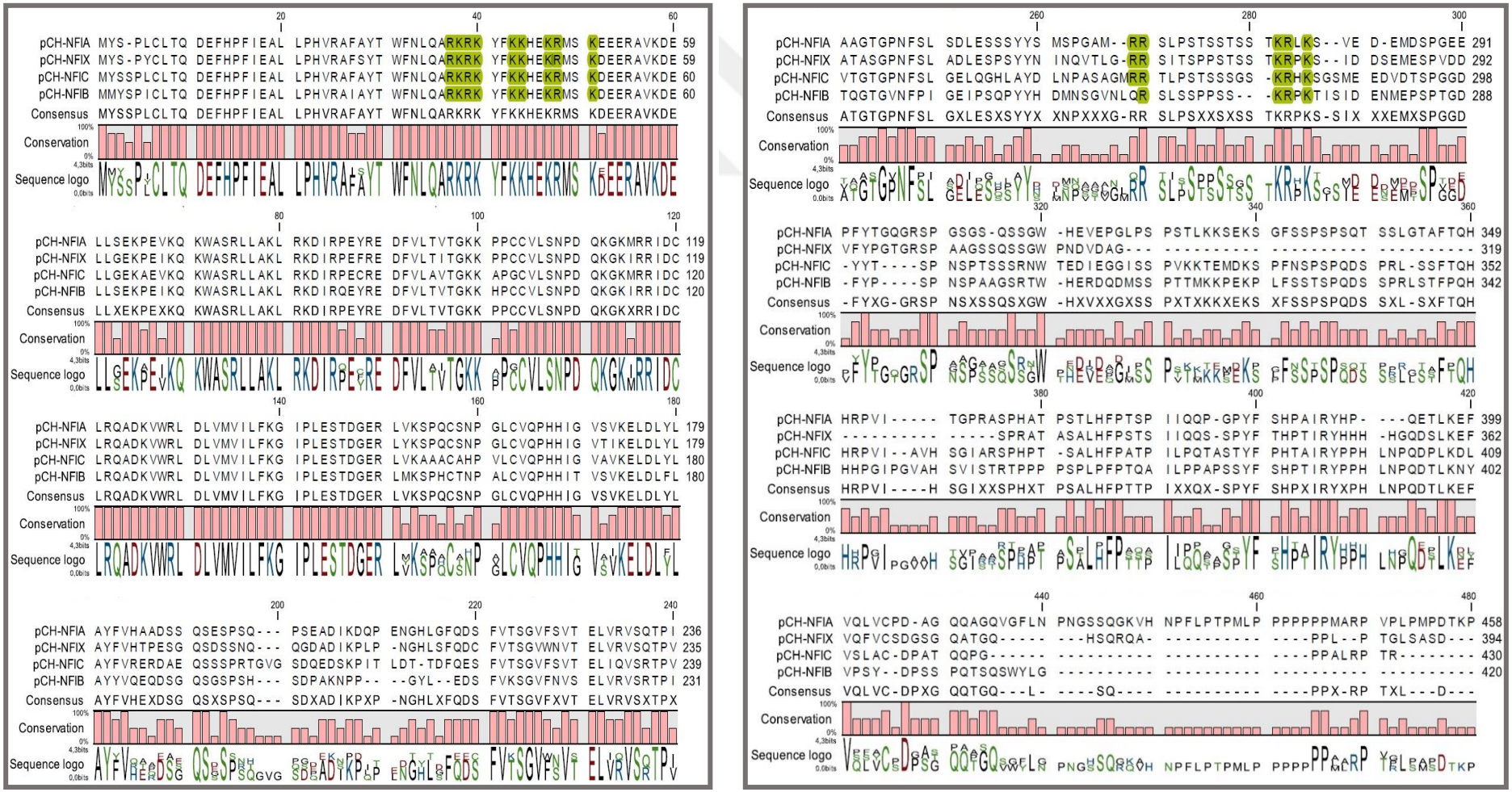

Figure 1.3: Protein sequence alignment of NFI isoforms produced in mouse. NFIA Isoform 4, NFIB Isoform 2, NFIC Isoform 2 and NFIX Isoform 2 were used for alignment. The first 210 amino acids constitute N-terminal domain, remaining amino acids belong to C-terminal domain. CLC Main Workbench 7 software were used to align sequences. Nuclear localization signals were labelled as green. NLS1 represents single basic type (constituted by more than four basic aa) and NLS2 represents bipartite basic type (basic amino acids are separated by 10-12 aa) of classes of nuclear localization signals.

1.1.1 Binding partners of NFIs

To date, several proteins have been identified to interact with NFI family members and regulate expression of target genes. Recently, FOXA1 was shown to be an interaction partner of NFI family (Grabowska et al., 2014). In this study, each member of NFI was shown to bind with FOXA1. N-terminal transactivation and forkhead domains of FOXA1 were shown to be important for interactions with NFIX. Additionally, in the presence of FOXA1, NFIX interacts with Androgen receptor (AR) and modulates AR mediated gene expression. Knockdown of NFIX in the prostatic cell line, LNCaP, led to upregulation of NFIA and NFIB suggesting that NFI members may compensate for and regulate expression of each other. Ski, which is a nuclear protooncogene, has been found to be another interaction partner of both NFIA and NFIB. Interaction with preformed NFI dimers is required for Ski to associate with DNA (Tarapore et al., 1997). In this study, formation of multimers constituted by NFI dimers and Ski results in transcriptional activation of NFI target genes.

NFI performs its regulatory role in glial differentiation by interacting with other transcription factors. NFIA induces differentiation of glial cells and is required for inhibition of neurogenesis and specification of glial fate in the central nervous system (Deneen et al., 2006). Olig2, a basic helix-loop-helix (bHLH) transcription factor citical for oligodendrocyte specification, interacts with NFIA. During differentiation of oligodendrocyte progenitors in the spinal cord, interaction of NFIA and Olig2 antagonizes pro-astrocytic function of NFIA which is required to induce oligodendrocyte differentiation (Deneen et al., 2006). NFIA mediates glial differentiation by interacting with Sox9 (Kang et al., 2012) and synergistic function of Sox9 and NFIA is antagonized by Olig2, which directly interacts with NFIA (Glasgow et al., 2014; Deneen et al., 2006). Presence of Olig2 facilitates NFIA and Sox10 interaction which leads to suppression of genes required for astrocyte commitment (Glasgow et al., 2014). Elsewhere, in muscle tissue, NFIX regulates myogenesis by interacting with Protein kinase C θ (PKCθ). During mammalian myogenesis, NFIX was shown to directly interact with PKCθ and MEF2A and acts as a bridge to link these proteins (Messina et al., 2010). Formation of a complex between NFIX, PKCθ and MEF2A allows, and enhances, PKCθ mediated phosphorylation and activation of MEF2A which induces fetal muscle specific gene expression (Messina et al., 2010).

Thus, NFI binds other transcription factors and regulatory proteins to regulate glial differentiation and myogenesis.

Phosphorylation status of NFI is important to induce gene expression from particular promoters. Weak interactions between Calcineurin, a serine/threonine phosphatase, and NFI was detected in malignant glioma cells, suggesting that Calcineurin might be one of the phosphatases responsible for hypo-phosphorylated status of NFI (Brun et al., 2013). Hypo-phosphorylated NFI increases expression of brain fatty acid binding protein (B-FABP) and glial fibrillary acidic protein (GFAP) implicating Calcineurin in regulation of NFI transcriptional activity and promoter occupancy.

Interaction of NFI with proteins involved in stress response regulates NFI mediated gene expression in glioblastoma cells. NFIX was found to regulate expression of genes related to heat stress, such as Heat shock factor 1 (HSF1) by interacting with high mobility group protein 1 (HMGN1) and CGG triplet repeat binding protein 1 (CGGBP1) (Singh et al., 2009). NFIX participates in a heat sensitive complex with transcriptional repressor CGGBP1 and HMGN1 which non-specifically binds to DNA leading to dissociation of histone H1 from nucleosomes resulting in open chromatin structure. Under chronic heat shock conditions, NFIX is recruited to HSF1 promoter by CGGBP1 and HMGN1 to suppress HSF1 expression.

Previously, NFI transcription family members were grouped into two categories depending on their ability to alter the level of basal transcription on PEPCK gene promoter (Crawford et al., 1998). NFIA and NFIB stimulate basal transcription from PEPCK promoter, while NFIC and NFIX inhibit transcription. Leahy et al.(1999) showed that NFI isoforms inhibit expression from PEPCK promoter when co-expressed with catalytic subunit of protein kinase A (PKA-c). It was determined that NFIC directly interacts with CREB Binding Protein (CBP). This interaction requires C-terminal transactivation domain of NFIC and CREB binding domain of CBP. NFIA was shown to block PKA-c and CREB mediated induction of PEPCK promoter by competing for CREB binding domain of CBP, suggesting that this competition maintains a low level of transcription from this promoter induced by cAMP (Leahy et al., 1999).

NFIA, NFIB, NFIC and NFIX were shown to interact with Regulatory Factor X1 (RFX1) in pituitary cells (Norquay et al., 2003). NFIs and RFX1 recognize and bind

to particular regions referred to as P sequence elements B (PSE-B) and P sequence elements A (PSE-A) within the promoter of Placental Choronic somatomammotrophins (CS), which belongs to human growth hormone gene family. The interaction between NFIs and RFX1 regulates repression of gene expression from the CS promoter (Norquay et al., 2003).

NFI mediated gene expression is regulated by TGF-β which induces activation of C-terminal transciptional activation or repression domain of NFI (Alevizopouluos et al., 1995). This regulation is not associated with phosphorylation of NFI. Instead, TGF-β regulates interactions of NFI and histone H3, one of the nucleosome components. NFI binds to H3 via its proline rich C-terminal domain, and this interaction induces transcriptional activity of NFI (Alevizopouluos et al., 1995). Finally, Pirin was isolated as an NFI interacting protein using a yeast two hybrid screen of HeLa cell cDNA library (Wendler et al., 1997).

1.2 Investigation of Putative NFI Interaction Partners

In order to identify proteins that may interact with NFIB in the developing central nervous system, a yeast two-hybrid (Y2H) screen was previously performed using a human fetal brain cDNA library (Yilmaz et al 2013). The Y2H system, which searches for protein-protein interactions, is based on activation of reporter gene expression driven by bait and prey interaction. Protein of interest is fused to Gal4 DNA Binding Domain (DBD) and is used as bait and cDNA library fused with Gal4 Activation Domain (AD) is used as prey. If an interaction occurs among bait and prey protein, DBD and AD reconstitute the Gal4 transcription factor that induces expression of reporter gene. Exploiting this system, where NFIB fused to DBD was used as bait to screen a human fetal brain cDNA library fused to AD, PIN1 and LMO4 were identified as putative NFIB interaction partners (Yılmaz, 2013). Interestingly, among 12 prey colonies that encoded a full or partial protein, 4 prey clones corresponded to PIN1 and 2 prey clones matched LMO4. The roles of these proteins in neural differentiation, their localization in the cell and during development, and their ability to regulate function of other transcription factors suggested that they are good candidates to investigate as potential interactors of NFIB, and could regulate NFIB function *in vivo*.

1.3 Protein Interacting with Never in Mitosis A (PIN1)

PIN1, was first discovered as an interaction partner of never in mitosis gene A (NIMA), an important mitotic kinase in *Aspergillus nidulans*, and is ubiquitously expressed in eukaryotes (Yeh and Means, 2007). PIN1 belongs to the peptidyl-prolyl isomerase family which catalyzes *cis-trans* isomerization of peptidyl prolyl bonds and changes conformation of its substrates (Yeh and Means, 2007). PIN1 consists of N-terminal WW domain (1-39 aa) and C-terminal PPIase domain (50-163 aa) linked by a short and flexible region (40-49 aa) (Figure 1.4) (Namanja et al.,2011). While the WW domain is responsible for recognition of serine-proline (SP) and threonine-proline (TP) residues, C-terminal PPIase domain has isomerase activity. Recognition motif was first described as a phosphorylated (serine/threonine) followed by a proline which is generated by proline directed kinases (such as cycline dependent kinases) and mitogen activated kinases (Shaw, 2007). However, phosphorylation independent interaction of PIN1 with CPEB and BPGAP1 has also been identified (Pan et al., 2009; Nechama et al., 2013).

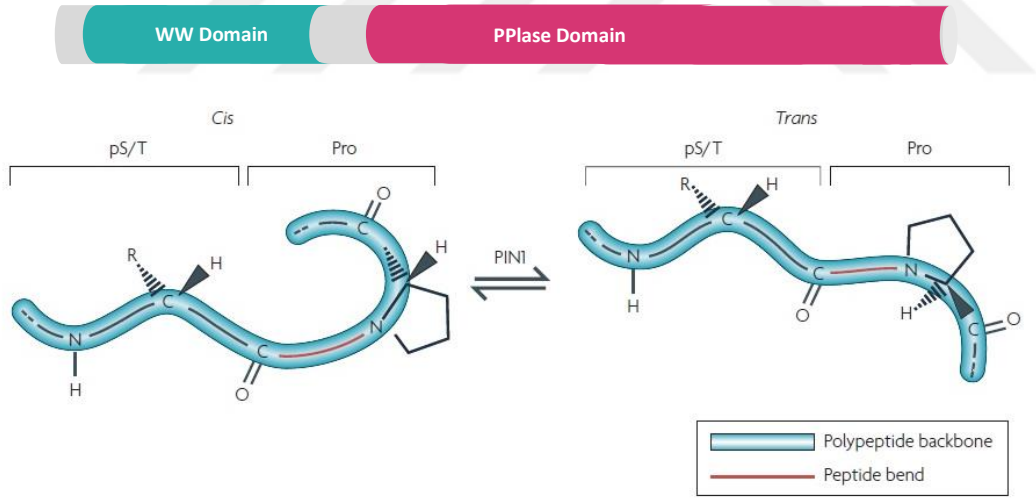

Figure 1.4: PIN1 structure and Cis-trans isomerization of prolyl bond is catalyzed by PIN1. Domain structure of PIN1 is shown in the upper panel. PIN1 consists of N-terminal WW domain and C-terminal PPIase domain. WW domain binds to phosphorylated serine or threonine residues followed by a proline. Catalytic activity of protein belongs to C-terminal PPIase domain. Cis-trans isomerization prolyl bond is shown in the lower panel. Isomerization of prolyl bond results in conformational change of target protein and alteration in function of substrate (adapted from Yeh and Means).

Numerous proteins are PIN1 targets, including DNA and RNA polymerases, protein and lipid kinases, as well as transcription factors (listed by Lu and Hunter, 2014). Consequences of interactions with PIN1 varies depending on the interaction partner. Target proteins may change their conformation due to isomerization by PIN1 which then influences their function. PIN1 may increase stability or induce degradation of some targets, or may affect their subcellular localization, phosphorylation status or ability to interact with other proteins (Nakamura et al, 2012; Yoon et al., 2014; Nakano et al., 2009). The overall consequences of PIN1 interactions are activation or repression of gene expression, increased or decreased cell proliferation and apoptotic or anti-apoptotic cellular responses (Lu and Hunter, 2014).

1.3.1 Regulation of cell differentiation by PIN1

PIN1 plays important roles during development as a regulator of cell differentiation in the CNS. Recently, PIN1 was found to interact with Gro/TLE1:Hes1 and form a multi protein complex with HIPK2 (Ciarapica et al., 2014). Hes1 had previously been shown to inhibit neurogenesis and had to be down regulated for neuronal and glial differentiation to proceed (Ishibashi et al., 1994). During cortical neurogenesis, PIN1 negatively regulates anti-neurogenic function of Gro/TLE1:Hes1 by associating with HIPK2. HIPK2 phosphorylates Gro/TLE1:Hes1 and creates binding sites for PIN1. Suppression of transcriptional repressor activity of Gro/TLE1:Hes1 is mediated by formation of this complex with HIPK2 and PIN1. Conformational changes in Gro/TLE1 lead to weakening of Gro/TLE1:Hes1 complex binding to DNA and suppresses its anti-neurogenic function (Ciarapica et al., 2014).

PIN1 expression is correlated with neural progenitor cell (NPC) differentiation in mouse embryos. In PIN1 KO mice, differentiation of late-born neurons is significantly inhibited leading to fewer upper layer neurons in the cortex (Nakamura et al, 2012). In this study, PIN1 was shown to interact with β-catenin in neural progenitor cells (NPCs). Interaction with PIN1 increases stability of β-catenin by regulating its conformation. After phosphorylation of β-catenin, PIN1 stabilizes its conformation in neural progenitor cells and induces neuronal differentiation (Nakamura et al, 2012).

PIN1 is also a positive regulator of osteoblast differentiation (Lee et al., 2013). It directly binds to phosphorylated Runx2 and positively regulates its transcriptional activity. Regulation is associated with increased stability of Runx2 due to suppression

of proteasomal degradation due to PIN1 binding. Additionally, PIN1 plays an important role in differentiation of fibroblasts into adipose cells. PIN1 directly interacts with phosphorylated CREB and suppresses its transcriptional activity. Inhibition of CREB activity by PIN1 interactions may be responsible for enhanced differentiation of fibroblasts into adipose cells (Uchida et al., 2012). In short, PIN1 binding is important for modulating function or stability of transcription factors and other proteins which regulate neuronal, osteoblast and adipose differentiation.

1.3.2 Regulation of protein stability by PIN1

Interestingly, PIN1 binding was shown to stabilize or destabilize SMADs, which like NFIs carry the MH1 domain, as well as other target proteins: PIN1 participates in a complex with SMAD2/3 after phosphorylation of SMADs via TGF-β signaling (Nakano et al., 2009). SMAD linker region between MH1 and MH2 domains contains four (S/T)P motifs that are putative targets of PIN1. Mutation analysis showed that all of four putative (S/T)P motifs in the linker region as well as the MH2 domain of SMAD3 are essential for this interaction. Additionally, protein levels of SMAD2/3 decrease in the presence of PIN1. PIN1 induces conformational changes in SMAD2/3 which enhances its interaction with Smurf2 proteins leading to SMAD2/3 ubiquitination and proteasomal degradation. Thus, TGF-β signaling is inhibited (Nakano et al., 2009). Moreover, PIN1 was also found to interact with another member of SMAD family, SMAD1/5. Interaction between SMAD1 and PIN1 occurs in a phosphorylation dependent manner. BMP2 signaling increases co-localization of PIN1 and SMAD1/5 in the nucleus. In the absence of PIN1, protein levels of SMAD1 decreases. Inhibition of PIN1 activity leads to increased ubiquitination of SMAD1 by Smurf1, and this event negatively regulates BMP signaling. Conformational change caused by prolyl isomerization makes SMAD1 inaccessible to Smurf1 and protects SMAD1 from degradation (Yoon et al., 2014).

In addition to its effects on SMAD stability, PIN1 can also destabilize a variety of proteins, including PML (Promyelocytic leukemia protein), a tumor supressor protein identified in acute promyelocytic leukemia patients, in the form of a fusion protein with retinoic acid receptor a (PML-RARa). PIN1 binds PML in a phosphorylation dependent manner (Reineke et al., 2008). Interaction of PIN1 leads to degradation of PML via ubiquitination mediated mechanism in MDA-MB-231 breast cancer cells (de

Thé, and Chen, 2010). SUMOylation of PML inhibits its interaction with PIN1, indicating that SUMOylation blocks PML degradation mediated by PIN1 (Reineke et al., 2008). PIN1 also affects PML- RARa stability and therefore may be a drug target in developing therapies for acute promyelocytic leukemia (Gianni et al., 2009; Wei et al., 2015).

HIPK2 is homeodomain interacting kinase whose activity is regulated by PIN1. HIPK2 regulates DNA damage induced apoptosis in cells. Activation of HIPK2 is associated with HIPK2 oligomerization and its site specific auto-phosphorylation which increases its kinase activity and pro-apoptotic efects. Auto-phosphorylation of HIPK2 creates new sites that are recognized by PIN1. Binding PIN1 protects HIPK2 from polyubiquitination by Siah-1 through a mechanism that interferes with HIPK2-Siah-1 interaction (Bitomskya et al., 2013). Thus, PIN1 regulates stability of target proteins by increasing their degradation, or protecting from degredation mechanisms.

1.3.3 PIN1 binding regulates gene expression

PIN binds directly to transcription factors or coactivators that regulate gene expression from target promoters. Heat shock factor 1 (HSF1) regulates expression of *hsp* (heat shock protein) genes. Stress induction leads to trimerization and translocation of HSF1 from cytoplasm to nucleus. PIN1 and phosphorylated HSF1 bind each other upon induction with heat shock stress and this interaction is required for efficient binding of HSF1 to DNA. In the absence of PIN1, expression of HSF1 target genes decreases (Wang et al., 2013).

PIN1 is also a coactivator of steroid receptor coactivator-3 (SRC-3) (Yi et al., 2005). PIN1 interacts with phosphorylated SRC-3 and increases progesterone receptor (PR) and estrogen receptor (ER) function and half-life of SRC-3. This interaction leads to conformational changes on SRC-3 that potentiate its interaction with other coregulators, such as CBP. SRC-3 interaction with PIN1 requires SRC-3's N-terminal bHLH domain, serine/threonine rich region and C-terminal CBP interaction domain including histone acetyl transferase activity (HAT). Together with SRC-3, PIN1 activates expression of both progesterone and estrogen response elements (Yi et al., 2005). In addition, PIN1 and c-Fos, which is a member Activating protein-1 (AP-1) family, bind to each other and regulate AP-1 dependent gene expression (Monje et al., 2005). Phosphorylation of c-Fos on its C-terminus by ERK in response to growth

factors is required for PIN1 binding and may contribute to elevated transcriptional activity of AP-1. Changes in conformation due to isomerization by PIN1 may enable c-Fos to associate with other partners (Monje et al., 2005).

PIN1 also interacts with phosphorylated histone 1 (H1) (Raghuram et al., 2013). Interaction between these two proteins leads to increased de-phosphorylation of H1. In the presence of PIN1, H1's affinity for DNA increases. PIN1 may also stabilize H1 binding to chromatin in the transcriptionally active sites (Raghuram et al., 2013).

1.3.4 Regulation of cell cycle by PIN1

During neuronal differentiation, neural progenitors exit the cell cycle and become postmitotic. Regulation of the cell cycle is important for controlling neurogenesis. PIN1 is known to be an important regulator of proper progression or inhibition of cell cycle (Shaw, 2007). PIN1 was shown to attenuate the activity of mitosis promoting factors and act as a negative regulator of mitotic activity in G2 phase (Lee et al., 2013). Aurora A and Bora, essential kinases involved in mitosis, phosphorylate several proteins that are required for G2/M activation and mitotic entry (Asteriti et al., 2015). Inhibitory function of PIN1 is suppressed by its interaction with Aurora A, which phosphorylates PIN1 at Ser16. Phosphorylation of PIN1 at Ser16 decreases its affinity for its substrates. This event leads to progression of the cell cycle. Phosphorylated Bora was found to be another interaction partner of PIN1. PIN1 alters localization of Bora, which results in its premature degradation. Down-regulation of Bora then leads to delay in mitotic entry. Mechanisms that involve Aurora A and Bora are both required for regulation of mitosis (Lee et al., 2013).

During murine embryonic development, PIN1 is required for progression of cell cycle (Atchison et al., 2003). In the absence of PIN1, primordial germ cells (PGCs) have a longer cell cycle and overall cell proliferation decreases. This in turn leads to severely reduction in number of germ cells produced (Atchison et al., 2003). Thus, PIN1 activity is required for proper progression of cell cycle, cell proliferation and cell differentiation.

1.4 LIM Only-4 (LMO4)

LIM proteins bind a variety of target proteins via their LIM domain and act as an adaptor that links proteins including transcription factors, cofactors, structural

proteins, kinases and other LIM domain proteins in multiprotein complexes. LIM proteins are classified into four different categories: LIM homeodomain (LIM-HD) proteins, LIM-only (LMO) proteins, LIM kinases and other LIM containing proteins. All members contain at least one LIM domain. The other domains found in LIM proteins such as the homeodomain, the kinase domain and the cytoskeletal binding domain confer unique features to each member (Bach, 2000). LIM Only proteins comprise two closely spaced LIM domains (Kadrmas and Beckerle, 2004). LIM domain is a conserved protein interaction domain found in eukaryotic organisms. It is composed of approximately 55 amino acids. Eight of these are conserved cysteine and histidine residues. Conserved cysteine and histidine residues at particular positions constitute tandem zinc-binding motifs in LIM domains (Figure 1.5). Zinc ions are bound by these conserved Cys-Cys-His-Cys residues (Kadrmas and Beckerle, 2004). Although the LIM domain is similar to the GATA-1 zinc finger motif, there is no evidence suggesting that the LIM domain actually binds DNA (Sum et al., 2002). Zinc finger motif of the LIM domain is an essential module for protein-protein interactions (Kenny et al., 1998; Gamsjaeger et al., 2007; Matthews et al., 2000).

LMO4 belongs to LIM-Only (LMO) subfamily of LIM proteins and was first discovered as LDB1 interacting protein. LMO4 contains two tandem LIM domains linked by a short linker and lacks other functional domains. No stable contacts were found between the two LIM domains (Deane et al., 2004). Interactions with binding partners, such as LDB1, fix orientation of LIM domains and stabilize its structure. Both LIM domains of LMO4 are essential for its interaction with LDB1. LDB1 binds to a single face of nuclear LIM proteins and allows simultaneous binding of another protein to the same LIM domain (Deane et al., 2004). While LMO proteins have no nuclear localization signal, they are localized in both the nucleus and the cytoplasm. Nuclear localization of LMO4 is associated with its interaction with LDB1 which has a nuclear localization signal itself, suggesting that LMO4 binds LDB1 and is localized in the nucleus (Kenny et al., 1998).

LDB1/NLI/Clim2 is a nuclear protein that interacts with LIM domain containing transcription factors. The N-terminal domain of LDB1 contains 200 amino acids responsible for dimerization while the C-terminal domain, also referred to as LIM interacting domain (LID), includes 38 residues which are sufficient for binding to the LIM domain (Jurata, 1997).

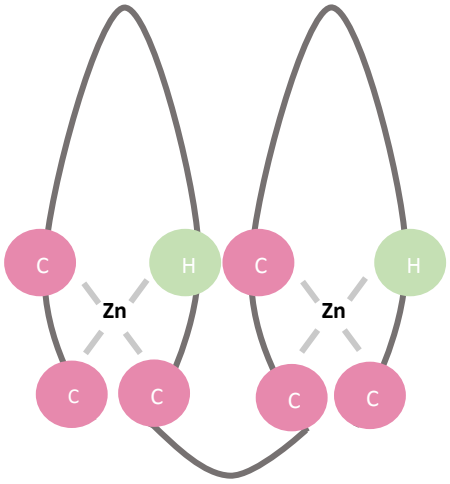

Figure 1.5: Topology of LIM domain. Tandem zinc binding motifs of LIM domain is shown. Pink and green circles indicate conserved cysteine (C) and histidine (H) residues, respectively.

1.4.1 Regulation of neuronal differentiation by LMO4

Severeal pieces of evidence indicate that LMO4 plays an important role in neuronal differentiation. LMO4 is expressed in the preplate, in the subventricular and ventricular zones in the cortex and in the hippocampus (Huang et al., 2009). LMO4 is required for neural tube development, proper neuronal differentiation in the cortex and establishment of projection neuron subtype diversity within the motor cortex (Huang et al., 2009; Asprer et al., 2011; Cederquist et al., 2013). Knock out studies show that absence of LMO4 leads to impaired neuronal differentiation in the cortex and disruption of molecular identity of neurons in the motor cortex (Asprer et al., 2011; Cederquist et al., 2013). In KO mice, cortex layers that form are thinner compared with the wild type cortex (Asprer et al., 2011). In addition, deletion of LMO4 leads to closure defects in the dorsal brain, disruption of specific cortical regional markers, size and shape of functional areas in cortex (Huang et al., 2009). Moreover, LMO4 regulates calcium dependent gene expression (Kashani et al., 2006). In the absence of LMO4, organization of pre- and post synapses is impaired in the barrel field of mice (Kashani et al., 2006).

LMO4 may regulate neuronal differentiation by forming complexes with bHLH transcription factor Neurogenin 2 (Asprer et al., 2011). In the embryonic cerebral cortex, LMO4 and LDB1 bind simultaneously to Neurogenin 2 (NGN2), which regulates neurogenesis and neuronal radial migration, and form a multiprotein complex (Asprer et al., 2011). LDB1 stabilizes and strengthens interactions between NGN2 and LMO4. This complex is recruited to E-box containing region within the

NGN2 target genes to stimulate their expression. Additionally, LMO4 regulates balanced generation of two distinct interneuron types, V2a and V2b, arising from the same progenitors in the spinal cord (Kimura, 2008). Generation of different cell fates is determined by LIM protein partners of LDB1 that participate in a multiprotein complex. In the presence of LMO4, competition of LMO4 with Lhx3 for binding to LDB1 suppress V2a cell fate. (Lee et al., 2008). Moreover, LMO4 induces V2b cell fate by interacting with SCL (Tal1) and Gata2 and NLI, linking these two transcription factors to each other (Joshi et al., 2009).

Snail family transcription factors are important regulators of neural crest development in vertebrates (Nieto et al., 1994). LMO4 interacts with both Slug and Snail of the Snail transcription factor family. This interaction requires the SNAG domain at the beginning of the Snail N-terminus, the complete Slug N-terminal domain, which is composed of SNAG domain and additional sequences, and LMO4 LIM domains (Ochoa et al., 2012). Binding of LMO4 contributes to Slug/Snail function and regulates neural crest development.

1.4.2 Binding partners of LMO4

To date, several proteins were found to interact with LMO4. LMO4 is capable of controlling transcription, both positively and negatively, depending on the cell context and its binding partners.

LMO4 interacts with nuclear receptors, cofactors or components of chromatin remodeling complex to modulate target gene expression (Singh, 2005; Sum et al., 2002). Specifically, LMO4 interacts with both Estrogen receptor α (ERα), metastasis tumor antigen 1 (MTA1), a corepressor protein of ERα, and Histone deacetylases (HDAC) to form a multiprotein complex. LMO4 represses expression of ERα target genes. Repression by LMO4 requires functional HDAC (Singh, 2005). Thus, LMO4 may behave as a linker or scaffolding protein to stabilize corepressor complex.

LMO4 is also involved in breast oncogenesis. LMO4 interacts directly with cofactor CtBP-interacting protein (CtIP) and BRCA1, which is a tumor suppressor protein and form a multi protein complex (Sum et al., 2002). LDB1 is also a component of these multi protein complexes. Interaction of LMO4 with BRCA1, suppresses transcriptional activity of BRCA1. These data suggest that LMO4 may function as a repressor of BRCA1 mediated transactivation in breast cancer.

Interactions between LMO4 and mouse Deformed Epidermal Autoregulatory factor-1 (mDEAF1) was first identified by Sugihara et al (1998). Although both LIM domains of LMO4 are required for this interaction, the second LIM domain of LMO4 and N-terminal domain of mDEAF1 are especially important (Sugihara et al., 1998; Joseph et al., 2014). LID domain of LDB1 competes with mDEAF1 for the binding face on LMO4 and thus prevents DEAF1 from binding to LMO4 binding (Joseph et al., 2014), suggesting that either LDB1 and DEAF1 binds the same face of LMO4 or LDB1 binding induces a conformational change in LMO4. LMO4 itself has no nuclear localization signal, its localization in the nucleus is facilitated by interacting partners containing a nuclear localization signal, such as LDB1. DEAF1 has both nuclear localization and nuclear export signals. Therefore, binding of DEAF1 to LMO4 facilitates nuclear localization of LMO4. LMO4 is in turn thought to increase nuclear localization of DEAF1 by sequestering its nuclear export signal (Joseph et al., 2014).

Interestingly, like PIN1, LMO4 also interacts with SMADs. SMAD DNA binding proteins translocate into nucleus when phosphorylated. As described earlier, SMADs consist of N-terminal MH1 and C-terminal MH2 domains (Miyazono, 2000). LMO4 interacts with several receptor-activated SMAD proteins. Remarkably, this interaction requires MH1, as well as linker domains of SMADs (Lu et al., 2006). LMO4 recruits SMADs to DNA to modulate TGF-β signaling.

1.5 Aim of the Study

As outlined above, PIN1 and LMO4, were previously identified as potential NFIB binding partners in a yeast two hybrid screen. Both proteins are expressed in the developing CNS, in the ventricular zones populated by neural stem cells and progenitors where NFIs are also expressed. Both are required for neural development and interact with and regulate transcription factors, including SMADs, transcription factors that also carry the MH1 domain found in NFIs. Here, we set out to investigate interactions between NFI transcription factor family members and PIN1 and LMO4. To test if NFIB putative binding partners could physically interact with NFIs *in vitro*, we performed co-immunoprecipitation experiments and GST pull down assays. In order to identify regions of interaction in NFIB, we created NFIB mutants which contain only the N-terminal domain, the C-terminal domain or point mutations and tested these *in vitro*.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

Equipment used in this study are listed in Appendix A.

2.1.2 Commercial Kits

Commercial kits used in this study are listed in Appendix B.

2.1.3 Chemicals

Chemicals used in this study are listed in Appendix C.

2.1.4 Enzymes and buffers

Enzymes and buffers used in this study are listed in Appendix D.

2.1.5 Growth and freezing media

2.1.5.1 Complete medium for HEK293T cell line

Complete medium is prepared by mixing 440 ml DMEM, 50 ml Fetal Bovine Serum (FBS), 5 ml Penicillin/Streptomycin (P/S) (100X), 5 ml L-Glutamine (L-Gln) (100X) and stored at 4^{o}C.

2.1.5.2 Antibiotic free medium for transfection with PEI

Antibiotic free medium is prepared by mixing 44.5 ml DMEM, 5 ml FBS, 0.5 ml L-Gln (100X) and stored at 4^{o}C.

2.1.5.3 Freezing medium for HEK293T cell line

Freezing medium is prepared by mixing 1 ml DMSO, 4 ml DMEM and 5 ml of FBS, sterilized with 0.22 um filter and stored at -20^{o}C.

2.1.6 Bacterial strains

2.1.6.1 Stellar competent cells

Chemically competent HST08 strain of *E. coli* (Clontech, PT5055-2) was used for transformation of In-Fusion Cloning reaction. Strain provides high transformation efficiency.

2.1.6.2 DH5α

Chemically competent DH5α strain of *E. coli* which is deficient in restriction and intracellular endonucleases was used for replication of plasmids to further use in overexpression studies.

2.1.6.3 BL21

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

2.1.7 Bacterial culture media

In this study, LB medium, LB agar medium, LBG medium, LBG agar medium, 2xYT medium, NZY broth were used to cultivate bacterial cells. Antibiotic was added to each of the medium after sterilization to obtain selective culture condition. Antibiotics used and their stock and final concentrations are listed in Table 2.1.

Table 2.1 : Stock and final concentration of antibiotics.

	Stock concentration	**Final concentration**
Ampicillin	100 mg/ml	100 ug/ml
Kanamycin	50 mg/ml	34 ug/ml
Chloramphenicol	34 mg/ml	50 ug/ml

2.1.7.1 LB medium

10 gr of tryptone, 10 gr of NaCl, 5 gr of Yeast extract were combined in 900 ml dH_2O. Components were dissolved by stirring, and volume was completed to 1 L. Medium was sterilized by autoclaving at 121^oC for 15 min.

2.1.7.2 LB-agar medium

10 gr of tryptone, 10 g of NaCl, 5 g of yeast extract and 15 g of agar were combined and dissolved in 900 ml dH_2O by stirring. Volume was completed to 1 L. Medium was sterilized by autoclaving at 121^oC for 15 min.

2.1.7.3 LBG medium

10 gr of tryptone, 5 g of NaCl and 5 g of yeast extract were combined and dissolved in 900 ml dH_2O by stirring. Volume was completed to 990 ml. Medium was sterilized by autoclaving at 121^oC for 15 min. After the medium cooled to 50^oC, 10 ml of sterile 2M glucose was added.

2.1.7.4 LBG-agar medium

10 gr of tryptone, 5 g of NaCl, 5 g of yeast extract and 15 g of agar were combined and dissolved in 900 ml dH_2O by stirring. Volume was completed to 990 ml. Medium was sterilized by autoclaving at 121^oC for 15 min. After the medium coolded to 50^oC, 10 ml of sterile 2M glucose was added.

2.1.7.5 2xYT

2xYT was prepared by dissolving 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl in 900 ml dH_2O by stirring. pH was adjusted to 7 and volume was completed to 1 L. Medium was sterilized by autoclaving at 121^oC for 15 min.

2.1.7.6 NZY broth

NZY medium was prepared by dissolving 10 g of NZ amine (casein hydrolase), 5 g of yeast extract, 5 g of NaCl in 900 ml dH_2O. pH was adjusted to 7.5 using NaOH. Medium was sterilized by autoclaving at 121^oC for 15 min. Filter sterilized 12.5 ml of 1M $MgCl_2$, 12.5 ml of 1M $MgSO_4$, 20 ml of 20% (w/v) and 10 ml of 2M glucose were added prior to use.

2.1.8 Solutions

2.1.8.1 2M Glucose

7,2 g of glucose (Mw: 180,16) was dissolved in total 20 ml dH_2O to obtain 2M glucose solution. It was sterilized by filtration through 0,2 μm filter. Solution was aliquoted and stored -20°C.

2.1.8.2 100 mM IPTG

IPTG was used to induce GST tagged protein expression in BL21 cells. 25 mg of IPTG was dissolved in 1 ml dH_2O to obtain 100 mM IPTG. Filter-sterilized IPTG was stored at -20°C.

2.1.8.3 $CaCl_2$ solution

$CaCl_2$ solution was used for preparation of chemical competent cells. Each components and their amount and final concentration are given in Table 2.2.

Table 2.2 : Formulation of $CaCl_2$ solution.

Components	Amount	Final concentration
PIPES	0,15 g	10 mM
$CaCl_2.2H_2O$	0,442 g	60 mM
Glycerol	7,5 ml	15%
dH_2O	Up to 50 ml	

2.1.9 Buffers used in SDS PAGE and western blotting

2.1.9.1 5X SDS sample buffer

Protein samples were mixed with 5X Sample Buffer prior to loading into the SDS Polyacrylamide gel. Usage of concentrated sample buffer enables to load larger volume of protein. Components and formulation of 5X Sample Buffer are given in Table 2.3.

Table 2.3 : Formulation of 5X Sample Buffer.

Components	Amount	Final concentration
0.5 M Tris-Cl	600 μl	300 mM
Glycerol	400 μl	40% (v/v)
SDS	0.1 gr	0.01% (w/v)
BPB	0.1 gr	0.01% (w/v)

2.1.9.2 1.5X SDS sample buffer

1.5X Sample Buffer was used for elution of proteins that bound to affinity resin. Components and formulation of 1.5X Sample Buffer are given in Table 2.4.

Table 2.4 : Formulation of 1.5X Sample Buffer.

Components	Amount	Final concentration
0.5 M Tris HCl	187.5 µl	93.75 mM
Glycerol	150 µl	15%
SDS (10% w/v)	300 µl	3% (v/w)
BPB (10 mg/ml)	75 µl	0.075 mg/ml
dH_2O	287.5 µl	

2.1.9.3 10X Running buffer

10X Running buffer was prepared by dissolving 30,2 gr Tris base (MW:121.14 g/mol) and 144 gr Glycine (MW:75.06 g/mol) in 800 ml dH_2O. Final volume was completed to 1 L.

2.1.9.4 1X Running buffer

1X Running Buffer was prepared by diluting of 10X Running Buffer. Components and formulation of 1X Running Buffer is shown in Table 2.5.

Table 2.5 : Formulation of 1X Running Buffer.

Components	Amount	Final concentration
10X Running Buffer	90 ml	1X
SDS (10%)	10 ml	0.1%
dH_2O	900 ml	

2.1.9.5 Blocking buffer

Blocking buffer was used to prevent non-specific binding of antibodies to the unbound sites of the membrane. Milk powder was used as blocking agent. 3 g of skim milk powder was dissolved in 100 ml TBS-T.

2.1.9.6 Ponceau S solution

Ponceau S solution was used for staining of nitrocellulose membrane to check transfer efficiency. Components and formulation of solution are shown in Table 2.6.

Table 2.6 : Formulation of Ponceau S solution.

Components	Amount	Final Concentration
Acetic acid	1 ml	1% (v/v)
Ponceau S	0.5 g	0.5% (w/v)
dH_2O	Up to 100 ml	

2.1.9.7 1.5 M Tris-HCl (pH: 8.8)

90,75 g of Tris base (MW: 121.14 g/mol) was dissolved in 400 ml dH_2O and pH was adjusted to 8.8. Volume was completed to 500 ml. Buffer was sterilized by autoclaving.

2.1.9.8 1 M Tris-HCl (pH 7,5)

60,57 g of Tris base (MW: 121.14 g/mol) was dissolved in 400 ml dH_2O and pH was adjusted to 7.5. Volume was completed to 500 ml. Buffer was sterilized by autoclaving.

2.1.9.9 0.5 M Tris-HCl (pH: 6.8)

30.3 g of Tris base (MW: 121.14 g/mol) was dissolved in 400 ml dH_2O and pH was adjusted to 6.8. Volume was completed to 500 ml. Buffer was sterilized by autoclaving.

2.1.9.10 10% SDS

10 g of SDS was dissolved in 100 ml dH_2O.

2.1.9.11 0.1% SDS

0.1% SDS was prepared by diluting of 10% SDS. 1ml of SDS (10%) was completed to 100 ml by adding dH_2O.

2.1.9.12 10% Ammonium persulfate

10 g of Ammonium persulfate was dissolved in 100 ml dH_2O.

2.1.9.13 Coomassie blue staining and de-staining solutions

Coomassie blue staining solution is used to stain protein bands separated by SDS-PAGE. Stain is removed by de-staining solution. Components and formulations of solution are given in Table 2.7.

Table 2.7 : Formulations of Coomassie blue staining and de-staining solutions.

Components	Staining		De-staining	
	Amount	**Final Concentration**	**Amount**	**Final Concentration**
Acetic acid	10 ml	10% (v/v)	10 ml	10% (v/v)
Methanol	50 ml	50% (v/v)	50 ml	50% (v/v)
Coomassie Brilliant Blue (G-250)	0.1 g	0.1(w/v)	-	-
dH_2O	Up to 100 ml		Up to 100 ml	

2.1.9.14 1X Transfer buffer

Components and formulation of 1X Transfer Buffer are given in Table 2.8.

Table 2.8 : Formulation of 1X Transfer Buffer.

Components	Amount	Final concentration
10X Running Buffer	100 ml	1X
Methanol	200 ml	20%
dH_2O	700 ml	

2.1.9.15 1X TBS and 1X TBS-T

Components and formulations of 1X TBS and 1X TBS-T solutions are shown in Table 2.9.

Table 2.9 : Formulations of 1X TBS and 1X TBS-T.

	TBS		TBS-T	
	Amount	**Final concentration**	**Amount**	**Final concentration**
1M Tris (pH 7.5)	50 ml	50 mM	50 ml	50 mM
5M NaCl	30 ml	150 mM	30 ml	150 mM
Tween20	-	-	1 ml	0.1%
dH_2O	Up to 1L		Up to 1L	

2.1.9.16 5M NaCl

58,44 grams of NaCl (MW: 58,44) was dissolved in 150 ml dH_2O and volume was completed to 200 ml.

2.1.10 Components of SDS-polyacrylamide gel

10%, 12% and 15% separating gels and 4% of stacking gel were used this study. Components and formulations are given in Table 2.10.

Table 2.10 : Formulations of separating and stacking gel used.

	% of Separating gel			% of Stacking gel
Components	**10%**	**%12**	**15%**	**%4**
Acrylamide solution	2,5 ml	3 ml	3,75 ml	0.5 ml
1.5M Tris-HCl (pH: 8.8)	2,5 ml	2.5 ml	2,5 ml	----
0.5M Tris-HCl (pH: 6.8)	----	----	----	1.25 ml
10% SDS	100 µl	100 µl	100 µl	50 µl
dH_2O	4.9 ml	4.5 ml	3.65 ml	3.2 ml
10% APS	100 µl	100 µl	100 µl	50 µl
Temed	4 µl	4 µl	4 µl	5 µl

2.1.11 Buffers for cell extraction

2.1.11.1 1X Sample buffer for whole cell extraction

1X Sample Buffer was used for whole cell extraction of Hek 293T. Components and formulation of 1X Sample Buffer are given in Table 2.11.

Table 2.11 : Formulation of 1X Sample Buffer.

Components	Amount	Final Concentration
0.5M Tris-HCl (pH: 6.8)	1.25 ml	62.5 mM
SDS (10% (w/v))	2 ml	2% (w/v)
Glycerol	1 ml	10% (v/v)
dH_2O	Up to 10 ml	

2.1.11.2 NP-40 Lysis buffer for membrane extraction

NP40 Lysis Buffer was used for lysis of cells to be analyzed for protein-protein interaction. Protease inhibitor cocktail was added freshly prior to use. Components and formulation of NP40 Lysis buffer is given in Table 2.12.

Table 2.12 : Formulation of NP40 Lysis Buffer.

Components	Amount	Final concentration
1M Tris-Cl (pH 7.5)	10 ml	50 mM
5 M NaCl	6 ml	150 mM
NP40	2 ml	1% (v/v)
0.5 M EDTA	2 ml	5 mM
dH_2O	Up to 200 ml	

2.1.12 Buffers used for agarose gel electrophoresis

2.1.12.1 50X TAE

242 g of Tris base (MW: 121.14 g/mol) was dissolved in 750 ml dH_2O, then 57.1 ml of glacial acetic acid and 100 ml of 0,5 M EDTA (pH:8) were added and volume was completed to total 1L.

2.1.12.2 1X TAE

1X TAE was prepared by diluting of 50X TAE. 20 ml of 50X TAE was combined with 980 ml dH_2O.

2.1.13 Antibodies

Antibodies used for western blot analysis and working dilutions are listed in Table 2.13. As a preservative, 0.02% sodium azide is added to working dilutions of antibody to protect from bacterial contamination.

Table 2.13 : Table Antibodies, supplier catalog numbers (Cat No) and working dilutions (WR).

Name of Antibody	Cat No	WR
Monoclonal Anti-Polyhistidine Clone HIS-1	Sigma H1029	1:1000
Polyclonal DYDDDDK Tag Antibody	CST 2368	1:500
HA-Tag monoclonal antibody Clone C29F4	CST 3724	1:1000
Myc-Tag monoclonal antibody Cone 9B11	CST 2276	1:1000
Anti-HA tag antibody polyclonal	Ab9110	1:1000
Anti-GST antibody polyclonal	Ab19256	1:1000
Pin1 (H-123) polyclonal	Sc-15340	1:200
Pin1 (G8)	Sc-46660	1:1000
Stabilized Peroxidase Conjugated Goat Anti-Rabbit (H+L)	Thermo Scientific, 32430	1:1000
Stabilized Peroxidase Conjugated Goat Anti-Mouse (H+L)	Thermo Scientific, 32460	1:1000
Veriblot for IP secondary antibody	Abcam, 131366	1:1000

2.1.14 Primers used for In-Fusion cloning and site directed mutagenesis

Primers used at PCR amplificarion steps of both In Fusion Cloning and Site Directed Mutagenesis were listed in Table 2.14.

Table 2.14 : List of primers used In Fusion Cloning and Site Directed Mutagenesis.

Primer Name	Sequence	Tm (oC)
LMO4_F	TACCGAGCTCGGATCACCACCATGGAGTACCCATACGATGTTCCAGATTACGCTCGAGAGATGGTGAATCCGGGCAGC	**60**
LMO4_R	GCCCTCTAGACTCGAAGGATCCTTTTAGCGGACCTTCTGGTC	
PIN1_F	CCAGATTACGCTCGAATGGCGGACGAGGAG	**60**
PIN1_R	TAGACTCGAAGGATCTCACTCAGTGCGGAG	
LBD1_F	CCAGATTACGCTCGAATGCTGGATAGGGAT	**55**
LBD1_R	TAGACTCGAAGGATCTTACTGGGAGGCCTG	
pGEX4T3_LMO4_F	GGTTCCGCGTGGATCCATGGTGAATCCGGGCAG	**54**
pGEX4T3_LMO4_R	GATGCGGCCGCTCGATTAGCAGACCTTCTGGTCTGGCAG	
pCMV-TdTomato_PIN1_F	ATGGACGAGCTGTACATGGCGGACGAGGAGAAG	**58**
pCMV-TdTomato_PIN1_R2	ATTGGCGCCGCGGCCTCACTCAGTGCGGAGGAT	
pCMV-TdTomato_LMO4_F	ATGGACGAGCTGTACATGGTGAATCCGGGCAGC	**61.2**
pCMV-TdTomato_LMO4_R	ATTGGCGCCGCGGCCTTAGCAGACCTTCTGGTCTGGCAG	
pEGFP-C2_NFIB3_F	GGACTCAGATCTCGAGCATGATGTATTCTCCCATC	**52**
pEGFP-C2_NFIB3_R	TAGATCCGGTGGATCCTAGCCCAGGTACCAGGA	
FLAG_PIN1_F	TACCGAGCTCGGATCCACCATGGAGGACTACAAAGACGATGACGACAAGTGTACAATGGCGGACGAGGAGAAG	**60,9**
FLAG_PIN1_R	GCCCTCTAGACTCGAGTCACTCAGTGCGGAGGAT	
FLAG_LMO4_F	TGACGACAAGTGTACAATGGTGAATCCGGGCAGC	**56,6**
FLAG_LMO4_R	AAACGGGCCCTCTAGATTAGCAGACCTTCTGGTCT	

Table 2.14 Cont.: List of primers used In Fusion Cloning and Site Directed Mutagenesis.

Primer Name	Sequence	Tm (oC)
pGEX4T3_LMO4_LID_F	ATGGCCATTTGAATTCACTTCAGAGCAATCCACTACTGCCAGACCAGAAGGTCTGCGGTGGTAGCGGTGGTCATATGGGTAGCGGTGGTGATGTG ATGGTGGTG	**51**
pGEX4T3_LMO4_LID_R	AGTCACGATGCGGCCTTACTCGTCGTCAATGCC	
pcDNA3.1_Nfib_C_F	CCAGATTACGCTCGAGGGGAGGACAGCTTTGTAAAATC	**58**
pcDNA3.1_Nfib_C_R	TAGACTCGAAGGATCCCTAGCCCAGGTACCAGGA	
pCHNFIB2_C_F	TATGCTGGGCCTCACGGGCCGGAGGACAGCTTTGTAAAATC	**60.1**
pCHNFIB2_C_R	AGCTATGCATAGATCTCTAGCCCAGGTACCAGGA	
mNFIB_S292A_S295A_S	GGAGACTTTTACCCCGCTCCAAATGCACCAGCTGCTGGAA	**55**
mNFIB_S328A_S	CACTGTTTAGCTCTACAGCTCCACAGGATTCTTCC	
mNFIB_S312A_S	GAACGAGATCAAGATATGTCTGCTCCAACTACAATGAAGAAGC	
mNFIB_S284A_S	AAAACTATATCTATAGATGAAAATATGGAGCCAGCTCCTACAGGAGACTTTT	
mNFIB_S284A_AS	AAAAGTCTCCTGTAGGAGCTGGCTCCATATTTTCATCTATAGATATAGTTTT	

2.2 Methods

2.2.1 Construction of mammalian expression vector using in-fusion cloning

To construct mammalian expression vector, we used In-Fusion® HD Cloning Kit (Clontech, 011614) designed to clone one or more DNA fragment into any vector. This ligation independent cloning method uses In-Fusion enzyme recognizing 15 bp overlapping sequence at both 5' and 3' ends of the PCR product and linearized vector to re-combine them. Overlapping sequences are created with addition of nucleotide sequences to the 5' end of each primer designed for the amplification of target sequence. PCR is performed to amplify target sequence with additional sequences at both ends which homologous to the ends of the linearized plasmid. The linearized vector is generated by restriction digestion. In-Fusion reaction is set up after purification and quantification of PCR product and linearized vector. Then, a part of the reaction is transformed into Stellar® competent cells. Several colonies are picked, re-streaked, and grown for isolation of plasmid DNA to check sequence of generated plasmid with both restriction digestion and sequencing.

2.2.1.1 Amplification of target sequence for in-fusion cloning

Q5 Hig Fidelity DNA Polymerase (Neb, MO491) or CloneAmp HiFi PCR Premix (ClonTech, 639298) were used to amplify target sequences. Components and formulations of reactions performed using CloneAmp and Q5 Hi-Fi are listed Table 2.15 and Table 2.16, respectively. Reaction steps for each enzyme are shown in Table 2.17. Amplification products were purified from agarose gel using gel extraction kit to be explained in section 2.2.2.

Table 2.15 : Reaction components and formulation using CloneAmp HiFi Premix.

	Amounts in		**Final**
	Negative Rxn	**Sample Rxn**	
CloneAmp HiFi Premix	12.5 µl	12.5 µl	50% (v/v)
Forward Primer (10 µM)	1.25 µl	1.25 µl	2 µM
Reverse Primer (10 µM)	1.25 µl	1.25 µl	2 µM
Template DNA (0,5 ng/µl)	-----	2 µl	1 ng
dH_2O	10 µl	8 µl	

Table 2.16 : Reaction components and formulation using Q5 Hi-Fi.

	Amounts in		Final
	Negative Rxn	**Sample Rxn**	
5X Q5 Buffer	5 µl	5 µl	20% (v/v)
Forward Primer (10 µM)	1.25 µl	1.25 µl	2 µM
Reverse Primer (10 µM)	1.25 µ	1.25 µl	2 µM
dNTPs (10 mM)	0.5 µl	0.5 µl	0.2 mM
Template DNA (0,5 ng/µl)	-----	2 µl	1 ng
Q5 Hi-Fi	0.25 µl	0.25 µl	1% (v/v)
dH_2O	10 µl	8 µl	

Table 2.17 : PCR steps using CloneAmp HiFi Premix and Q5 Hi-Fi. Annealing temperatures were given for each primer sets on Table 2.13.

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

2.2.1.2 Preparation of linearized vector for in-fusion cloning

Double digestion was performed to linearize expression vectors for all In-Fusion reactions. Reactions were set up in a buffer in which enzymes exhibit the most activity and incubated appropriate temperature for 2 hr. Sequential digestion was performed for enzymes not exhibiting >50% activity in the same buffer. Reaction was set up for the enzyme that needed lowest salt concentration. When incubation step was completed, salt concentration was adjusted for the reaction needed by second enzyme. Second enzyme was added to the reaction and incubated at appropriate temperature. For instance, pcHNFIB2 vector digested with SfiI and BglII restriction enzymes that need different incubation temperature and salt concentration. Vector was digested first with SfiI enzyme for 1 hr in a low salt buffer (Buffer G) and incubated at 50°C for 1 hr. Then, high salt buffer was added and salt concentration was adjusted to 100 mM using 2 µl of 0.5M NaCl. Finally, BglII was added and incubated at 37°C for 1 hr. After each restriction digestion reaction, linearized vector was purified from agarose gel using gel extraction kit to be explained in section 2.2.2.

2.2.1.3 In-fusion cloning reaction

Cloning and negative control reactions were prepared according to manufacturer's instructions. Components and amount of each component used for cloning and negative control reaction are shown in Table 2.18. Reactions were set up and mixed. After mixing, tubes were incubated at 50°C for 15 min, then placed on ice. Reactions were stored at -20°C or used for transformation.

Table 2.18 : Components of In-Fusion cloning reaction.

Rxn Component	Cloning Rxn	Negative Control Rxn
Purified PCR fragment	100 ng	---
Linearized vector	100 ng for 0.5- to 10 kb 50 ng for <0.5 kb	1 µl
5X In-Fusion HD Enzyme Premix	2 µl	2 µl
Deionized water	Up to 10 µl	Up to 10 µl

2.2.1.4 Construction of pcDNA3.1-HA-LMO4 expression vector

cDNA encoding LMO4 was cloned into pcDNA3.1 vector using In-Fusion cloning kit. To obtain linearized vector, pcDNA 3.1 (+) was digested with BamHI and XhoI restriction enzymes. HA tagged LMO4 was generated by amplification of pGAL-LMO4 vector using primers named as LMO4_F and LMO4_R listed in Table 2.14. In addition to gene specific sequence of LMO4 and homolog sequence to linearized vector that are both required for amplification of target and then recombination of vector and amplified product, additional sequences were rationally inserted within the primers. Kozak consensus sequence was added to forward primer to initiate translation of target protein in mammalian cells efficiently, HA tag sequence was added to create target protein with a common affinity purification tag on its N-terminus. Additionally, to simplify further In-Fusion reactions, BamHI recognition sequence was completed between sequences coding HA tag and LMO4, and XhoI recognition sequence was generated within the downstream sequence of LMO4 on the reverse primer. Linearized pcDNA 3.1 vector restricted with XhoI and BamHI and PCR product of LMO4 were purified from agarose gel and In-Fusion cloning reaction was set up. 100 ng of linearized vector and 50 ng of purified LMO4 PCR product were used for the reaction. After transformation of Stellar competent cells with 2 µl of cloning reaction, a few colonies obtained from positive plate were re-streaked, grown overnight at 37°C in 5

ml LB medium containing Ampicillin. Plasmid DNA isolation was done for selected colonies using miniprep kit and sequence of generated plasmid was checked with both restriction digestion and verified by sequencing. XbaI recognition site within the multiple cloning site was not destroyed.

2.2.1.5 Construction of pcDNA3.1-HA-LBD1 expression vector

LBD1 cDNA was cloned into pcDNA3.1 vector using In-Fusion cloning kit. To obtain linearized vector including Kozak and HA tag sequences, previously designed pcDNA3.1-HA-LMO4 vector was digested with BamHI and XhoI restriction enzymes. LBD1 was amplified from pOBT-LBD1 vector using primers named as LBD1_F and LBD1_R listed in Table 2.14. Both linearized vector and amplified LBD1 product were purified from agarose gel using extraction kit and In-Fusion cloning reaction was set up. 60 ng of linearized vector and 50 ng of PCR product of LBD1 were used for the In-Fusion cloning reaction explained before. Sequence of constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.6 Construction of pcDNA3.1-HA-PIN1 expression vector

PIN1 gene was cloned into pcDNA3.1 vector with a similar method to construction of pcDNA3.1-HA-LBD1. Previously designed pcDNA3.1-HA-LMO4 vector was digested with BamHI and XhoI restriction enzymes. PIN1 gene was amplified from pGAL-PIN1 vector using primers named as PIN1_F and PIN1_R listed in Table 2.14. 60 ng of linearized vector and 50 ng of amplified product of PIN1 were used for In-Fusion reaction. Constructed plasmid DNA sequence was checked with restriction digestion and verified by sequencing.

2.2.1.7 Construction of pcDNA3.1-Flag-PIN1 expression vector

To obtain PIN1 protein with an alternative affinity purification tag on its N-terminus, PIN1 was designed with Flag affinity tag and cloned into pcDNA3.1 vector. In addition to PIN1 specific sequence and homolog sequence to linearized vector of each primer, Kozak consensus sequence and Flag tag coding sequence were added within the forward primer. BsrGI recognition sequence was also added between Flag tag and gene specific sequence to make further In-Fusion reactions easier. PIN1 was amplified from previously created pcDNA3.1-HA-PIN1 vector using primers listed in Table 2.14

and named as FLAG_PIN1_F and Flag_PIN1_R. PIN1 PCR product and pcDNA3.1 vector digested with BamHI and XhoI were purified from agarose gel. 100 ng of linearized vector and 100 ng of PCR product were used for In-Fusion reaction. Constructed plasmid DNA sequence was checked with restriction digestion and verified by sequencing.

2.2.1.8 Construction of pcDNA3.1-Flag LMO4 expression vector

In order to express LMO4 protein in mammalian cells with an alternative affinity purification tag on its N-terminus, LMO4 was designed with Flag affinity tag on its N-terminus and cloned into pcDNA3.1 vector. Previously designed pcDNA3.1-Flag-PIN1 expression vector was used as backbone and digested with BsrGI and XbaI. LMO4 was amplified from pGEX4T3-GST-LMO4 vector, to be explained later, using primers listed in Table 2.14 named as FLAG_LMO4_F and FLAG_LMO4_R. Amplification product and vector digested with BsrGI and XbaI were purified from agarose gel. 100 ng of linearized vector and 100 ng of PCR product were used for In-Fusion reaction. Sequence of constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.9 Construction of pcDNA3.1-HA-NFIB2-TAD expression vector

C-terminus of NFIB was cloned into pcDNA3.1 vector with a similar method to construction of pcDNA3.1-HA-LDB1 and pcDNA3.1-HA-PIN1 vectors. Previously designed pcDNA3.1-HA-LMO4 vector was digested with BamHI and XhoI restriction enzymes. C-terminus trans activation/repression domain of NFIB was amplified from pCHNFIB2 vector by using primers listed in Table 2.14 named as pcDNA3.1_Nfib_C_F and pcDNA3.1_Nfib_C_R. 100 ng linearized vector and 100 ng of PCR product were used for In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.10 Construction of pCH-HA-NFIB2-TAD expression vector

C-terminus of NFIB was cloned into pCH vector which is an alternative backbone to pcDNA3.1 for expression in mammalian cell. pCHNFIB expressing vector were sequentially digested with SfiI and BglII C-terminus of NFIB was amplified from pCHNFIB vector by using primers listed in Table 2.14 named as pCHNFIB2_C_F and pCHNFIB2_C_R. Amplification product and vector digested with SfiI and BglII were

purified from agarose gel. 100 ng linearized vector and 100 ng of amplified C-terminus of NFIB product were used for In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.11 Construction of pGEX4T3-GST-LMO4 expression vector

To express LMO4 in *E.coli* for GST Pull down experiments, cDNA encoding LMO4 cloned into pGEX4T3 bacterial expression vector. BamHI and XhoI were used for digestion of pGEX4T3 vector. LMO4 was amplified from pRK5-Myc-LMO4 vector which was a gift from Anirvan Ghosh Lab (Addgene plasmid # 22965) using primers listed in Table 2.14 named as pGEX4T3_LMO4_F and pGEX4T3_LMO4_R. Digested vector and PCR product were purified from agarose gel. 100 ng of vector and 100 ng of amplified product were used In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.12 Construction of pGEX4T3-GST-LMO4-LID expression vector

To express soluble form of LMO4 in *E.coli*, LMO4 was designed to fuse with LID domain of LBD1 on its C-terminus and cloned into pGEX4T3 vector. Previously designed pGEX-GST-LMO4 vector was digested using EcoRI and NotI. LID domain coding region was amplified from pcDNA3.1-HA-LDB1 vector using primers named as pGEX4T3_LMO4_LID_F and pGEX4T3_LMO4_LID_R listed in Table 2.14. Digestion of vector by EcoRI caused to loss of a part of coding region of LMO4, because of that, lost region of LMO4 was completed with addition of sequence within the forward primer. Digested plasmid and amplified product were purified from agarose gel. 100 ng of digested vector and 100 ng of PCR product were used for In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.13 Construction of pEGFPC2-NFIB expression vector

To express fluorescently tagged NFIB in mammalian cells, NFIB was cloned into pEGFPC2 vector. pEGFPC2 was digested with XhoI and BamHI. NFIB was amplified from pCHNFIB vector using primers listed in Table 2.14 named as pEGFP_C2_NFIB3_F and pEGFP_C2_NFIB3_R. 100 ng of vector and 100 ng of PCR product were used for In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.14 Construction of tdTomato-LMO4 expression vector

To express LMO4 protein with an fluorescent tag on its N-terminus, LMO4 was cloned into pCSCMV:tdTomato which was a gift from Gerhart Ryffel (Addgene plasmid #30530). BsrGI and NotI restriction enzymes were used to digest tdTomato vector. LMO4 was amplified from pRK5-Myc-LMO4 vector using primers listed in Table 2.14 named as pCMV_TdTomato_LMO4_F and pCMV_TdTomato_LMO4_R. 100 ng of vector and 100 ng of PCR product were used for In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.15 Construction of tdTomato-PIN1 expression vector

To express PIN1 protein with a fluorescent tag on its N-terminus, PIN1 was cloned into tdTomato vector. PIN1 was amplified from pcDNA3.1-HA-PIN1 vector by using primers listed in Table 2.14 named as pCMV_TdTomato_PIN1_F and pCMV_TdTomato_PIN1_R2. 100 ng of vector and 100 ng of PCR product were used for In-Fusion reaction. Constructed plasmid DNA was checked with restriction digestion and verified by sequencing.

2.2.1.16 Other expression vectors

Bacterial expression plasmid encoding for GST tagged Pin1 was a gift from Michael Yaffi (Addgene Plasmid #19027). Bacterial expression plasmid encoding for LDB1 was obtaned from Harvard Medical School (Clone ID: HsCD00322468). Mammalian expression plasmids encoding for HA tagged NFIA, NFIB, NFIC and NFIX were gifts from Richard Gronostajski (Addgene plasmids # 31404, 31405, 31403, 31402, respectively).

2.2.2 Gel Purification of PCR products or linearized vectors

Amplification product and linearized vector were purified from agarose gel using NucleoSpin Gel and PCR Clean Up Kit (Macherey-Nagel, 740609) or GeneJet Gel Extraction Kit (ThermoScientific, K0692). DNA fragment was extracted from agarose gel and gel weight was determined. For each 1 mg of gel, 2 µl of NTI buffer was added and gel pieces was solubilized by incubating on 50°C for 10 min. Up to 700 µl sample was loaded into NucleoSpin Gel and PCR Clean-up Column placed previously in a 2 ml collection tube and centrifuged for 30 sec at 11000xg. Flow-through was discarded. Loading step was repeated if necessary. 700 µl Buffer NT3 was added to the column

to wash and centrifuged for 30 sec at 11000xg. Flow-through was discarded. Washing step was repeated to improve A_{260}/A_{280} value. Silica membrane was dried by centrifugation at 11000xg for 1 min to remove Buffer NT3 completely. Then, column was placed in a fresh 1.5 ml micro centrifuge tube and incubated 5 min at 70°C. 20 µl Elution Buffer preheated previously to 70°C was added onto the center of the membrane, incubated for 3 min at room temperature and centrifuged for min at 11000xg. Elution step was repeated by adding 10 µl of preheated elution buffer. Quality and quantity of DNA was checked measurement by UV spectroscopy and agarose gel electrophoresis.

2.2.3 Agarose gel electrophoresis for detection of PCR products of vectors

1 gram of agarose was dissolved in 100 ml of 1X TAE by boiling with microwave to prepare 1% agarose gel. PronaSafe (Conda, CK13) was added before pouring into the gel cassette to make DNA bands visible. Samples were loaded into wells along with marker and run for 40-50 min at 90V.

2.2.4 Miniprep preparation

Plasmid DNA Purification Kit (Macherey-Nagel, 740588.50) or Genejet Plasmid Miniprep Kit (Thermo Scientific, K0503) was used to isolate plasmid DNA from small scale bacterial cell culture. 5 ml of starter culture for high copy plasmid (pcDNA3.1, pCH, pRK5, pEGFP backbones) or 10 ml of starter culture for low copy plasmid (pGEX backbone) was grown at 37°C overnight and harvested by centrifugation at 11000xg for 30 sec. Supernatant was discarded. 250 µl of Buffer A1 was added and pellet was re-suspended by vortexing. 250 µl of Buffer A2 was added and mixed by inverting the tube 6-8 times. Tube was incubated at room temperature for up to 5 min. 300 µl of neutralization solution was added and mixed thoroughly by inverting the tube for 6-8 times and centrifuged for 10 min at 11000xg. Up to 750 µl of supernatant was loaded onto the column and centrifuged for 1 min at 11000xg. Flow-through was discarded. 500 µl of Buffer AW was added to the silica membrane and centrifuged for 1 min at 11000xg. Flow-through was discarded. Then, 600 µl of Buffer A4 was added to the membrane and centrifuged for 1 min at 11000xg. Flow-through was discarded. Silica membrane was centrifuged for 2 min at 11000xg to remove residual wash buffer. Column was transferred to a fresh 1.5 ml tube and 25 µl of elution buffer was added to the membrane, incubated for 1 min at room temperature and centrifuged for 2 min

at 11000xg. Elution step was repeated with additional 25 µl elution buffer. Quality and quantity of plasmid DNA was checked by UV spectroscopy and by restriction digestion. Eluted plasmid was stored at -20°C.

2.2.5 Maxiprep preparation

GeneJET Plasmid Maxiprep Kit (Thermo Scientific, K0492) or NucleoBond®Xtra Midi/Maxi Kit (Mecherey-Nagel, 740414.50) was used for isolation of plasmid DNA.

2.2.5.1 Maxiprep using GeneJET plasmid maxiprep kit

Bacterial culture was grown overnight at 37°C and harvested by centrifugation at 5000xg for 10 min at +4°C. Supernatant was discarded. Pellet was re-suspended in 6 ml of resuspension solution including RNase A by vortexing until no cell clumps remain. 6 ml of Lysis solution was added and mixed gently by inverting the tube 4-6 times until the solution becomes viscous. Solution was incubated for 3 min at room temperature. 6 ml of Neutralization Solution was added and mixed by inverting the tube 5-8 times. 800 µl of Endotoxin binding reagent was added and mixed by inverting the tube 5-8 times. Then, tube was incubated at room temperature for 5 min. 6 ml of 96% ethanol was added to tube and mixed by inverting the tube 5-6 times. Then, centrifuged at 3200xg for 60 min. Supernatant was transferred to a fresh 50 ml tube by decanting. 6 ml of 96% ethanol was added and mixed by inverting the tube 5-6 times. Part of the sample (approximately 20 ml) was transferred to the column and centrifuged for 3 min at 2000xg. Flow-through was discarded. Loading step of sample to the column and centrifugation were repeated if necessary. 8 ml of wash solution I was added to the column and centrifuged for 2 min at 3000xg. Flow-through was discarded. 8 ml of wash solution II (including ethanol) was added to the column and centrifuged for 2 min at 3000xg. Washing and centrifugation steps were repeated. Tube was centrifuged for 5 min at 3000xg to remove residual wash solution. Column was transferred to a fresh 50 ml tube (provided). 1 ml of elution buffer was added to the center of the column, incubated for 2 min at room temperature, and centrifuged for 5 min at 3000xg. Quality and quantity of plasmid DNA was checked by UV spectroscopy and restriction digestion. Eluted plasmid was stored at -20°C.

2.2.5.2 Maxiprep using NucleoBond®Xtra midi/maxi kit

MN NucleoBond Xtra Maxi Plus Kit was used for maxiprep preparation. Bacterial culture was grown overnight at 37ºC and harvested by centrifugation at 3000xg for 20 min at +4ºC. Supernatant was discarded. Pellet was resuspended in 12 ml Resuspension Buffer by vortexing until no cell clumps remain. 12 ml Lysis Buffer was added and mixed by inverting the tube for 5 times. Mixture was incubated for 5 min at room temperature. The column filter was equilibrated by applying the equilibration buffer onto the rim of the column to wet completely. 12 ml Neutralization buffer was added to suspension and mixed until sample become colorless by inverting the tube 10 times. Sample was applied to the equilibrated column and waited for clarification of lysate by gravity flow. Column filter was washed with 15 ml equilibration buffer and filter was removed. The column was wash with 25 ml Wash Buffer. Plasmid DNA was eluted by adding 15 ml Elution Buffer and collected in a 50 ml tube. Then 10.5 ml isopropanol was added for precipitation of DNA, mixed thoroughly and incubated for 2 min at room temperature. Syringe was attached to Nucleobond® Finalizer and filled with mixture. Sample passed through the Finalizer drop wise manner, and flow-through was discarded. Finalizer was washed with 5 ml 70% ethanol, then dried by pressing air through Finalizer as strongly as possible for at least 6 times. Finalizer was placed on a fresh 1.5 ml micro centrifuge tube and plasmid DNA was eluted with 500 µl Tris or dH_2O. First eluate was transferred back into the syringe and eluted into the same tube a second time. Quality and quantity of plasmid DNA was checked by UV spectroscopy and restriction digestion. Eluted plasmid was stored at -20ºC.

2.2.6 Preparation of calcium competent cells

Frozen stock of bacterial cells (DH5α or BL21) were streaked onto LB agar plate without antibiotic and incubated overnight at 37ºC. After that, single colony was selected and inoculated into 5 ml LB medium without antibiotic. Culture was grown overnight at 37ºC on a shaker. Starter culture was inoculated into 100 ml LB medium without antibiotic and grown until OD_{600} reached to 0,5-0,6. When the OD_{600} reached to 0,5-0,6, culture was transferred into 50 ml tubes pre-chilled to +4ºC. Tubes were incubated on ice for 10 min, then centrifuged at 1600xg for 7 min at +4ºC. Supernatant was discarded. Pellets in both tubes were re-suspended in 10 ml ice-cold $CaCl_2$ for each and centrifuged for 5 min at 1600xg at 4ºC. Supernatant was removed. Pellets in

both tubes were re-suspended in 10 ml ice-cold $CaCl_2$ for each and tubes were incubated on ice for 30 min. then, tubes were centrifuged at 1600xg for 5 min at 4°C. Supernatant was removed. Pellets in both tubes were re-suspended in 2 ml ice-cold $CaCl_2$ for each and cells were aliquoted into 1.5 ml micro centrifuged tubes pre-chilled to 4°C.

2.2.7 Transformation

2.2.7.1 Transformation of DH5α

DH5α competent cells were used for transformation of mammalian expression vectors. LB medium and agar plates were preheated to 37°C. DH5α competent cells were thawed on ice just before use and 50 µl of cells were aliquoted directly into pre-chilled tube. 20 ng of plasmid DNA was added to tube mixed gently and incubated on ice for 30 min. Heat shock was performed at 42°C for 1.5 min and reaction was incubated on ice for 2 min. 450 µl of pre-heated LB medium was added to cells to complete the volume to 500 µl. Cells were incubated at 37°C, for 1 hr on a shaker, and plated on agar plate containing appropriate antibiotic.

2.2.7.2 Transformation of BL21

BL21 competent cells were used for GST pull down experiments to transform pGEX vectors. LB medium and agar plates containing glucose were preheated to 37°C. BL21 competent cells were thawed on ice just before use and 50 µl of cells were aliquoted directly into pre-chilled tube. 100 ng of plasmid DNA were added to tube, mixed gently and incubated on ice for 30 min. Then, heat shock was performed at 42°C for 1.5 min and reaction was incubated on ice for 2 min. 450 µl of preheated LB medium was added to cells to complete the volume to 500 µl. Cells were incubated at 37°C, for 1 hr on a shaker, and plated on LB agar plate containing 20 mM glucose and appropriate antibiotic.

2.2.7.3 Transformation of stellar competent cells

Stellar™ Competent Cells (Clontech, PT5055-2) were used for transformation of In-Fusion reactions. LB agar plates and SOC medium were pre-heated to 37°C. Competent cells were thawed on ice just before use. After that, cells were mixed gently by pipetting and 50 µl of cells were moved to a pre-chilled 1.5-ml micro centrifuge

tube. 2.5 µl of In-Fusion reaction were then added, mixed and incubated on ice for 30 min. Cells were heat shocked at 42°C for 60 sec and tubes were placed on ice for 1-2 min. 450 µl of SOC medium pre-warmed to 37°C was added to each tube to bring the final volume to 500 µl. Cells were incubated at 37°C for 1 hr on shaker. Then cells were plated onto LB agar medium containing selective antibiotic and incubated overnight at 37°C.

2.2.7.4 Transformation of NEB5α competent cells

NEB5α Competent cells (NEB, C2987) were used for transformation of XL site directed mutagenesis reactions. LB agar plates and SOC medium were pre-heated to 37°C. Competent cells were thawed on ice just before use. After that, cells were mixed gently by pipetting and 50 µl of cells were moved to a pre-chilled 1.5-ml micro centrifuge tube. 2 µl of DpnI treated mutagenesis reaction were then added, mixed and incubated on ice for 30 min. Cells were heat shocked at 42°C for 30 sec and tubes were placed on ice for 5 min. 950 µl of SOC medium pre-warmed to 37°C was added to each tube to bring the final volume to 1000 µl. Cells were incubated at 37°C for 1 hr on shaker. Then cells were plated onto LB agar medium containing selective antibiotic and incubated overnight at 37°C.

2.2.8 Bacterial growth for plasmid preparation

Single colony from freshly streaked plate were selected to inoculate 5 ml of LB medium containing selective antibiotic and incubated for 16 hours at 37°C while shaking at 200-225 rpm. Starter culture was used for miniprep preparation or diluted (1:1000 to 1:10,000) and inoculated to 250 ml LB medium containing appropriate antibiotic to precede maxiprep preparation.

2.2.8.1 BL21 growth and induction

Two colonies transformed with pGEX expression vector were selected and grown in 3 ml 2xYT medium individually until the OD_{600} reach 0,6-0,8. One of the colonies was induced by adding 2 µl 100 mM IPTG to obtain 0.1 mM final concentration. 1 ml of culture was harvested 1h after induction and another 1 ml of culture was harvested 2h after induction to decide sufficient induction time for over expression, cultures were centrifuged at 11000xg for 30 sec. Supernatant was discarded. Pellets were boiled within SDS sample buffer at 95°C.

2.2.9 Analysis of protein expression

2.2.9.1 Preparation of whole cell extracts

48 hours after transfection of HEK293T cells, whole-cell extracts were prepared using 1X Sample Buffer. Sample Buffer was heated to 95^{o}C before use. Transfected cells were washed twice with 1 ml PBS, and scraped with 400 µl 1X Sample Buffer. Lysates were boiled at 95^{o}C for 10 min, passed through a syringe for 5 times, and then cleared by centrifugation at 12000x*g* for 10 min. Supernatants were collected and aliquoted. Concentrations of each sample were determined by using BCA protein assay kit (Pierce), and then subjected to Western Blotting.

2.2.9.2 Membrane extraction

48 hrs after transfection of HEK293T cells, medium was aspirated and plate was washed two times with 1 ml PBS. Then, plate was placed on ice and 1 ml prechilled NP40 Lysis Buffer included EDTA and protease inhibitor was added. After incubation on ice for 30 min, plate was scraped and lysate was collected in a 1.5 ml pre-chilled micro centrifuge tube. Lysate was centrifuged for 10 min at 10000xg at +4^{o}C. Supernatant was aliquoted into fresh pre-chilled 1.5 ml tubes and stored at -80^{o}C.

2.2.9.3 Protein extraction from BL21 cells

Cells were harvested from liquid culture in a 1.5-ml micro centrifuge tube by centrifugation at 11000xg for 30sec. Pellet were re-suspended in 1/5 culture volume BugBuster® (Novagen, 70584-3). Cell suspension was incubated on rotator for 20 min at room temperature. Insoluble fraction was removed by centrifugation at 16,000xg for 20 min at 4^{o}C. Supernatant were transferred to a new tube and aliquoted for further use. Pellet were re-suspended in 1X Sample Buffer and heated at 95^{o}C for 5 min, then stored at -20^{o}C.

2.2.10 Determination of protein concentration

Pierce BCA Protein Assay Kit (Thermo Scientific, 23227) was used for quantitation of total protein. BCA Protein Assay is highly sensitive and selective method based on reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium. Chelation of two molecules of BCA with one molecule of cuprous ion (Cu^{+1}) leads to formation of purple colored product which absorbs light at 562 nm. It has been reported that peptide

bonds and presence of cysteine, cysteine, tryptophan and tyrosine residues in sample to be responsible for color formation. Bovine Serum Albumin was used as standard protein to prepare dilution series of known concentration. Preparation of known concentration of BSA is given in Table 19. 50 parts of BCA Reagent A and 1 part of BCA Reagent B were used to prepare working reagent (WR). 10 µl of each BSA standard and unknown sample were loaded into a microplate well as duplicates (sample to working reagent ratio=1:20, working range=125-2000µg/ml). 200 µl BCA WR was added to each well and mixed. Covered plate was incubated humidified box at 37ºC for 60 min. After that, plate was cooled down to room temperature. Then, absorbance was measured at 562 nm on a plate reader.

Table 2.19 : Preparation of BSA Standards.

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

2.2.11 SDS-PAGE gel electrophoresis

Appropriate amount of protein sample was mixed with 5X Sample Buffer and 20X DTT, then boiled at 95ºC for 5 min. After mixing, samples were loaded into wells along with a protein standard and started running at constant 90 V, then separated at constant 110 V.

2.2.12 Western blotting

When SDS-PAGE was completed; gel, nitrocellulose membrane and Whatman papers were equilibrated with 1X Transfer Buffer. Proteins were transferred to a 0,45 µm pore-size nitrocellulose membrane overnight at 4ºC in 1X Transfer Buffer at constant 16 V. After blotting, membrane was stained with Ponceau S to check transfer efficiency. After washing a few times with dH_2O until it becomes clear, membrane was blocked with 3% milk-powder in TBST at room temperature for 1 hour on shaker, then washed with TBST. Membrane was incubated with primary antibody diluted in

10% FBS in TBST overnight at 4°C on a rotator. Following day, membrane was washed 3 times with TBST for 5 min. Then, membrane was incubated with appropriate secondary antibody prepared in 3% milk-powder in TBST for 1 hour at room temperature on shaker and washed 2 times with TBST, and once with TBS for 5 min. Bands were visualized using chemiluminescent substrate.

2.2.13 Immunoprecipitation

To immunoprecipiate epitope tagged proteins of interest, we used monoclonal α-HA conjugated agarose (Sigma, A2095), c-Myc Monoclonal Antibody-Agarose Beads (Clontech, 631208) and α-Flag® M2 affinity gel (Sigma A2220). For each immunoprecipitation sample, 20 µl 1:1 suspension of the antibody conjugated agarose resin was transferred to 1.5 ml micro centrifuge tube. Resin in each tube was washed with 1 ml NP40 Lysis Buffer by inverting the tube and centrifuged for 30 sec at 12000xg. Liquid was discarded. Washing step was repeated twice. Appropriate amount (0.5-1 mg) of Hek 293T cell lysate was added to settled resin, and incubated overnight on an orbital shaker at +4°C. The following day, sample was centrifuged at 2000xg for 2 min at +4°C and supernatant was removed to a fresh tube, and stored at -20°C. Washing step was performed with 1 ml NP40 Lysis Buffer by inverting the tube and centrifuged 2000xg for 2 min at +4°C. Washing step was repeated for three times. Supernatant was completely removed after the last centrifugation and 50 µl 1.5X Sample Buffer was added into each tube. Immunoprecipitated proteins were denatured by boiling at 95°C for 5-10 min within the 1.5X Sample Buffer. Then, centrifuged at maximum g for 1 min at room temperature. Supernatant was removed into a fresh tube. DTT is added to sample if needed. Supernatant was stored at -20°C or resolved by SDS-PAGE.

2.2.14 GST pull-down

To pull down GST or GST tagged proteins, we used Glutathione-Agarose (Sigma, G4510) or Glutathione Sepharose 4B (GE 170756-01). Each resin was previously rinsed three times with BugBuster. Finally, prepared as 20% slurry in BugBuster and stored at 4°C. For each pull down assay, 50 µl glutathione resin (20% bed volume) was placed into a 1.5 ml microcentrifuge tube. Appropriate amount (10-20 µg) of BL21 cell lysate including GST or GST tagged proteins were incubated on glutathione resin for 50 min at room temperature on an orbital shaker. Then, Hek 293T cell lysate were

added onto resin, and incubated 2 hrs at 4ºC on an orbital shaker. Incubated mixure was centrigufed at 500xg for 5 min at 4ºC. Flow through was tranferred into a new tube. Pellet was washed three times with PBS including 1% TritonX (v/v). After the last washing step, 50 µl 1.5X Sample Buffer was added into tube, and pellet was boiled at 95ºC for 5-10 min. Finally, tube was centrifged at max g for 1 min. Eluate was trasferred into a new tube and analysed with western blotting.

2.2.15 Thawing, subculturing and freezing of HEK293T cells

2.2.15.1 Thawing of HEK293T cells

Frozen vial of HEK293T were thawed quickly in a 37ºC water bath. Cells were removed from vial into a fresh 15 ml tube. 9 ml of pre-warmed medium was added onto cells in a drop wise manner. Cells were centrifuged for 5 min at 300xg. Supernatant was removed. Pellet was re-suspended in remaining medium by tapping the tube. 10 ml fresh pre-warmed medium was added and cells were plated into 100 mm plate dish. Plate was placed into a 37ºC incubator with 5% CO_2.

2.2.15.2 Subculturing of HEK293T cells

Growth medium was aspirated, and plate was washed with 4 ml pre-warmed PBS. 2 ml of Tyrpsin EDTA was added onto cells, and incubated a few minutes until cells completely detached from plate surface. 8 ml of fresh pre-warmed medium was added for inactivation of Tyrpsin. Cells were collected into a 15 ml fresh tube and centrifuged at 300xg for 5 min. Supernatant was removed. Pellet was re-suspended in remaining medium by tapping the tube. Fresh medium was added into tube and cells were plated into new 100 mm plates at 1:5 ratio.

2.2.15.3 Freezing of HEK293T cell line

A half of the confluent HEK293T cell plate was washed with PBS and cells were detached by adding Tyrpsin EDTA described in section 2.2.15.2. After centrifugation, pellet was re-suspended in 1 ml of Freezing medium and transferred into a cryo tube. Tube was placed into isopropanol tank and stored at -80oC for short period. Cells should be stored at liquid nitrogen for longer period.

2.2.16 Transfection

2.2.16.1 Transient transfection of HEK293T cell line with X-tremeGene HP transfection reagent

$3x10^4$ cells/cm^2 were plated into culture vessels 24 hours before transfection. On the day of transfection, DNA and X-tremeGene HP transfection reagent was vortexed before using. DNA, diluent and X-tremeGene HP transfection reagent (Roche) were equilibrated to room temperature and reagent was vortexed briefly before use. DNA was diluted in serum free DMEM to obtain a final concentration of 1 µg DNA/100 µl medium and mixed. Transfection reagent was added directly into the medium containing diluted DNA at 1:3 ratio. DNA, reagent, diluent complex were then incubated at room temperature for 15 min. After incubation, complex was added to cultured cells in a dropwise manner. Plate was swirled to ensure distribution of complex over the whole surface of plate. Cells were incubated for 48 hrs. required amounts of DNA, diluent and transfection reagent for 6 well and 100 mm plate were listed in Table 2.19.

Table 2.20 : DNA, diluent and reagent amounts to be used in transfection with Xtreme-Gene HP.

Culture Vessel	Surface Area (cm2)	Amount of DNA	Amount of transfection complex to add each well	Final amount of transfection reagent (1:3 ratio)
6 well (1 well)	9.4	2 µg	200 µl	6 µl
100 mm	55	10 µg	1000 µl	30 µl

2.2.16.2 Transfection of HEK293T cell line with PEI

Branched PEI (1mg/ml) Sigma was used for transfection of HEK293T cell line. $3x10^4$ cells/cm^2 were plated into culture vessels 24 hours before transfection. On the day of transfection, growth medium was aspirated and plate was washed with 4 ml PBS. 6 ml fresh medium without antibiotic was added into plate. For 100 mm plate, 10 µg DNA was diluted in 100 µl DMEM. Then 30 µl PEI was added directly into the medium and tube was mixed immediately. After 15 min incubation, DNA-PEI transfection complex was diluted by adding 600 µl DMEM. Mixture was added into culture vessel in a dropwise manner. Plate was rocked to ensure distribution of complex over the whole surface of plate. 4 hrs after transfection, medium was aspirated and plate was washed

with 4 ml pre-warmed PBS. 10 ml of fresh medium was added into plate and cells were incubated for 48 hrs in a 37ºC incubator with 5% CO_2.

2.2.17 Site directed mutagenesis of NFIB

QuikChange Multi Site-Directed Mutagenesis Kit (Agilen, 200515) and QuikChange II XL Site Directed Mutagenesis Kit (Agilent, 200521) were used to make desired substitution mutation(s) on plasmid DNA coding for NFIB. For each strategy, mutated double stranded DNA was synthesized, parental DNA was digested using DpnI. Finally, a portion of the reaction mixture was transformed into chemically competent cells. pcHNFIB2 plasmid DNA was used as template DNA. Primers used for mutagenesis were listed in Table 2.14. Reaction components for each protocol listed in Table 2.21 and Table 2.22 were mixed and cycling protocols followed for each kit were given in Table 2.23, and Table 2.24. After temperature cycling, tubes were placed on ice for 2 mins. 1 µl DpnI (10 u/µl)was added into each tube and mixed. Tube included DpnI incubated at 37ºC water bath for 1 hr. 1.5 µl of multi site-directed mutagenesis reaction, 2 µl of XL site-directed mutagenesis reaction was used for transformation. For multi sire directed mutagenesis reaction DH5α chemically competent cells were used, whereas for XL site-directed mutagenesis reaction NEB5α competent *E.coli* (C2987) cells were used. Transformation protocols were described at section 2.2.7.1 and 2.2.7.4.

Table 2.21 : Reaction components and amount used in QuikChange Multi Site-Directed Mutagenesis.

Reaction Component	Template ≤5 kb	Template >5 kb
10X QuikChange Multi Rxn Buffer	2,5 µl	
dH_2O	Up to 25 µl	
Quik Solution	-	0+0,75 µl
dsDNA template	50 ng (x µl)	100 ng (x µl)
Mutagenic primers	X µl (100 ng each primer 1-3 primer)	
dNTP mix	1 µl	
QuikChange Multi Enzyme Blend	1 µl	

Table 2.22 : Reaction components and amount used in QuikChange XL Site-Directed Mutagenesis.

Reaction Component	Amount
10X Rxn Buffer	5 µl
dH_2O	Up to 50 µl
Quik Solution	3 µl
dsDNA template	10 ng
Mutagenic primers	125 ng of each primer
dNTP mix	1 µl
Pfu Ultra HF DNA Polymerase (2.5U/ µl)	1 µl

Table 2.23 : Cycling parameters for QuikChange Multi Site Directed Mutagenesis.

Temperature	Time	Cycles
95°C	1 min	30
95°C	1 min	
55°C	1 min	
65°C	9 min	

Table 2.24 : Cycling parameters for QuikChange XL Site Directed Mutagenesis.

Temperature	Time	Cycles
95°C	1 min	18
95°C	50 sec	
55°C	50 sec	
68°C	5 min	
68°C	7 min	

3. RESULTS

In order to investigate interactions between NFIs and putative NFIB binding partners LMO4 and PIN1, epitope tagged NFIs and prey proteins PIN1 and LMO4 were co-expressed in HEK293T cells, and subjected to co-immunoprecipitation experiments. Additionally, we performed GST pull-down assays using bacterially expressed GST-fusion prey proteins and NFI overexpressing HEK293T cell lysates. To identify NFI domains involved in interaction with prey proteins, we generated NFIB mutants that comprise only the NFIB N-terminal DNA binding and dimerization domain or C-terminal transactivation/repression domain and tested these in both co-immunoprecipitation and GST pull-down experiments. Moreover, some of the potential PIN1 recognition motifs on NFIB were mutated and assayed for PIN1 binding via co-immunoprecipitation and GST pull-down assays. Finally, NFIs were subjected to phosphatase treatment to investigate phosphorylation dependency of NFI-PIN1 interactions.

3.1 Construction of Expression Vectors

In order to overexpress NFI interacting proteins in cell culture, we first cloned cDNAs encoding PIN1 and LMO4 from pGADT7 yeast expression vector into the pcDNA 3.1 mammalian expression vector by homologous recombination. Following the cloning, we checked for efficiency of protein expression from vector constructs. HEK293T cells were transfected and lysates were analyzed by western blotting. According to western blot analyses, epitope tagged PIN1, when expressed in HEK293T cells, migrates at ~19 kDa whereas endogenous PIN1 migrates at ~17 kDa. Since LMO4 mammalian expression constructs failed to express this protein at the desired levels, we obtained the vector expressing Myc-tagged LMO4 (Addgene #22965). The band that migrates at ~22 kDa corresponds to Myc-tagged LMO4. Cloning of cDNA encoding NFIs with N-terminal His-tag were previously performed (Güler, 2016). Constructs expressing HA-tagged NFIs were acquired (Addgene #31404, 31405, 31403, 31402). Epitope tagged NFIA, NFIB, NFIC and NFIX migrate at ~60 kDa, ~47

kDa, ~58 kDa, ~48 kDa, respectively. LDB1 was cloned into mammalian expression vector with an N-terminal HA tag and used as positive control for co-immunoprecipitation experiments. The band corresponds to LDB1 migrates ~44 kDa.

For GST pull-down assays, LMO4 was cloned into pGEX-4T3 bacterial expression vector. Additionally, the vector encoding LMO4-LID fusion protein was generated in order to isolate LMO4 as soluble form. Both fusion proteins were expressed with an N-terminal GST fusion protein and when expressed in bacteria migrate at ~42 kDa and ~46 kDa, respectively. The band corresponding to GST-PIN1 migrates ~45 kDa. GST protein alone was used as negative control for pull down experiments and migrates at ~26 kDa.

In order to express NFIB, PIN1 and LMO4 with a fluorescent tag, cDNA encoding NFIB was cloned into pEGFP-C1 vector which contains the N-terminal EGFP tag. Additionally, LMO4 and PIN1 were individually cloned into pCSCMV:tdTomato vector to express these proteins with an N-terminal Tomato tag.

When introduced into the appropriate host organism, these vectors expressed recombinant proteins of the correct size. Target proteins fused with epitope tags or GST were used in both co-immunoprecipitation and GST pull-down assays. Vectors expressing fluorescent fusion proteins will be further used to determine localization of the target protein inside the cell.

3.2 Analysis of NFIs and PIN1 Interactions by Co-Immunoprecipitation Assays

In order to investigate potential interactions between NFIs and PIN1, HEK293T cells were transfected with plasmids expressing His-tagged NFIA, NFIB, NFIC or NFIX along with HA-tagged PIN1. Overexpressed proteins are some of the most abundant proteins in the cell lysate, therefore, we could detect non-specific binding between either overexpressed proteins and/or between overexpressed protein and antibody used for immunoprecipitation of that proteins. Therefore, in order to check for specifity of the interaction between target proteins, NFIB was co-expressed with MAD2B which was previously tested and used as negative control for co-immunoprecipitation experiments. α-His antibody was used for immunoblotting of membrane to detect His-tagged NFIs which co-immunoprecipitated with PIN1. Co-immunorecipitation studies showed that His-tagged NFIA, NFIC and NFIX could be co-immunoprecipitated with

HA-tagged PIN1 (Figure 3.1, Figure 3.2 and Figure 3.3), when expressed individually, none of them immunoprecipitated non-specifically with α-HA conjugated agarose resin. However, His-tagged NFIB precipitated non-specifically with α-HA conjugated agarose resin in the absence of HA-tagged PIN1 (Figure 3.4).

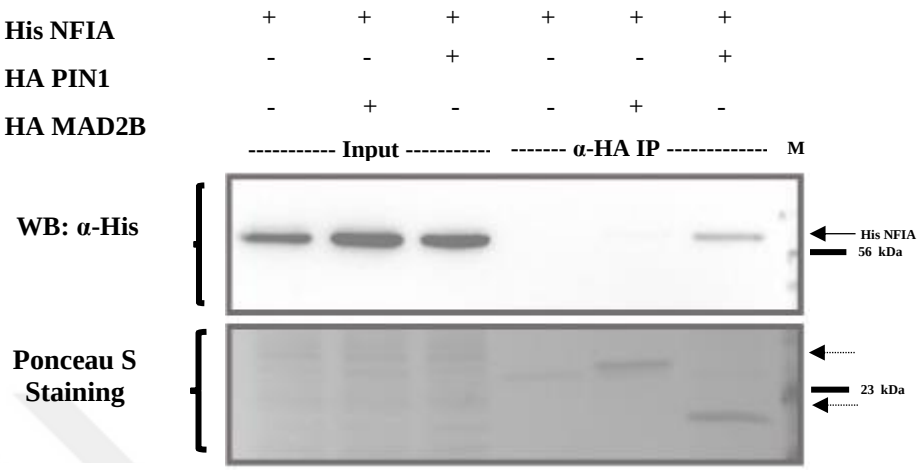

Figure 3.1 : Co-immunoprecipitation analysis of His-tagged NFIA and HA-tagged PIN1. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of HA tagged PIN1 and HA-tagged MAD2B using α-HA antibody followed by western blot analysis of His-tagged NFIC. Immunoprecipitates are shown together with 10 µg of each input lysate. The position of NFIA immunoreactive band is indicated with arrow. Membrane was visualized by Ponceau S Staining (Lower panel). Bands correspond to immunoprecipitated HA-tagged MAD2B and HA-tagged PIN1 are indicated with dashed arrows. M: Marker.

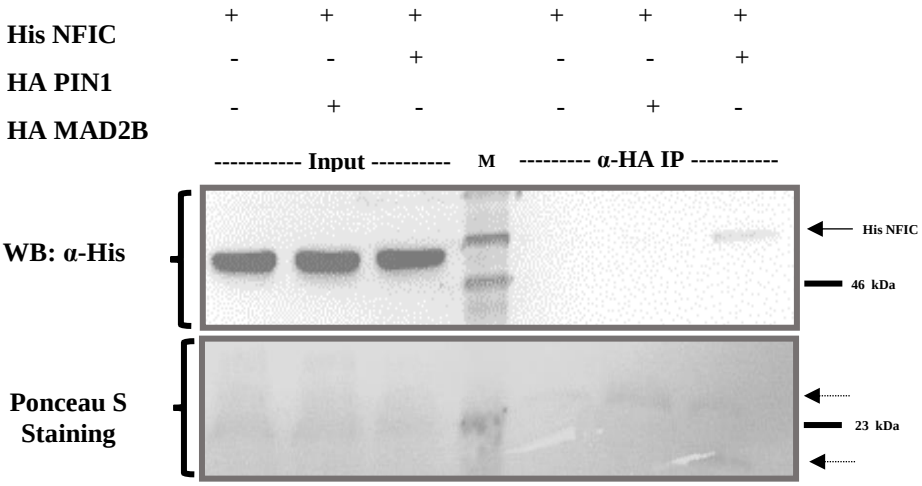

Figure 3.2 : Co-immunoprecipitation analysis of His-tagged NFIA and HA-tagged PIN1. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of HA-tagged PIN1 and HA-tagged MAD2B using α-HA antibody followed by western blot analysis of His-tagged NFIA. Immunoprecipitates are shown together with 10 µg of each input lysate. The position of NFIA immunoreactive band is indicated with arrow. Membrane was visuilized by Ponceau S Staining (Lower panel). Bands correspond to immunoprecipitated HA-tagged MAD2B and HA-tagged PIN1are indicated with dashed arrows. M: Marker.

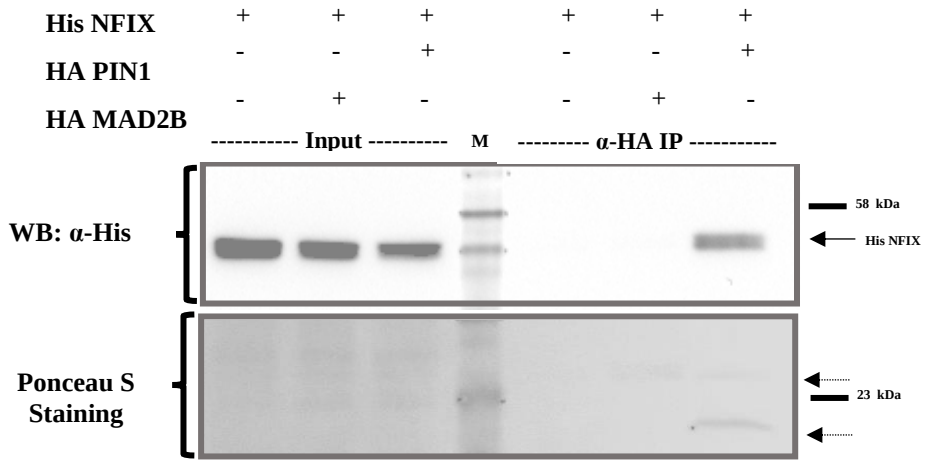

Figure 3.3 : Co-immunoprecipitation analysis of His-tagged NFIX and HA-tagged PIN1. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of HA tagged PIN1 and HA-tagged MAD2B using α-HA antibody followed by western blot analysis of His-tagged NFIX. Immunoprecipitates are shown together with 10 µg of each input lysate. The position of NFIX immunoreactive band is indicated with arrow. Membrane was visualized by Ponceau S Staining (Lower panel). Bands correspond to immunoprecipitated HA-tagged MAD2B and HA-tagged PIN1 are indicated with dashed arrows.M: Marker.

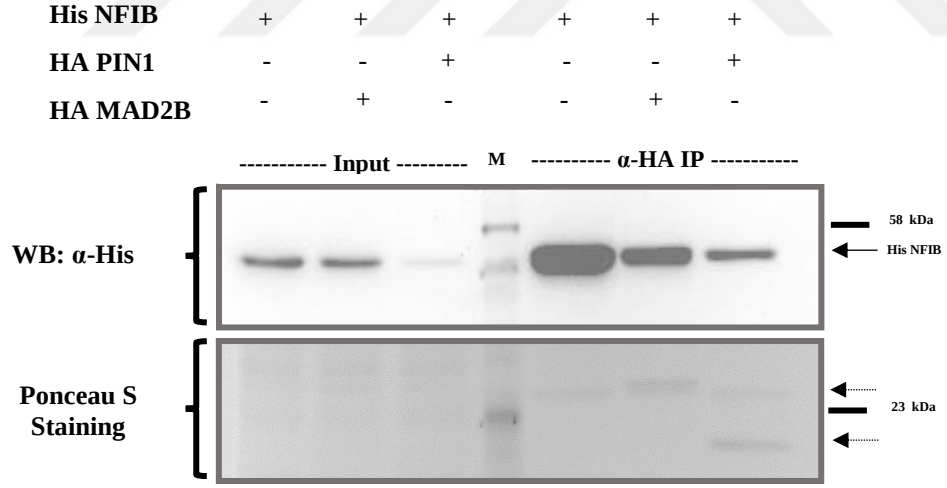

Figure 3.4 : Co-immunoprecipitation analysis of His-tagged NFIB and HA-tagged PIN1. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of HA-tagged PIN1 and HA-tagged MAD2B using α-HA antibody followed by western blot analysis of His-tagged NFIB. Immunoprecipitates are shown together with 10 µg of each input lysate. The position of NFIB immunoreactive band is indicated with arrow. Membrane was visualized by Ponceau S Staining (Lower panel). Bands correspond to immunoprecipitated HA-tagged MAD2B and HA-tagged PIN1are indicated with dashed arrows.M: Marker.

These results indicate that NFIA, NFIC and NFIX could co-immunoprecipitate with PIN1 in HEK293T cells when they are co-expressed. While His-tagged NFIB does co-immunoprecipitate with HA-tagged PIN1 when co-expressed with HA-tagged PIN1, it also precipitates with α-HA conjugated resin in the absence of any HA-tagged proteins. In order to overcome this artifact, we used different epitope tags on overexpressed proteins. Flag-tagged PIN1 was overexpressed in HEK293T cells along with HA-tagged NFIB. Immunoprecipitation assay performed using α-Flag conjugated resin showed that HA-tagged NFIB did not precipitate non-specifically with α-Flag conjugated agarose resin. Moreover, NFIB did co-immunoprecipitate with Flag-tagged PIN1 protein while HA-tagged MAD2B did not (Figure 3.5).

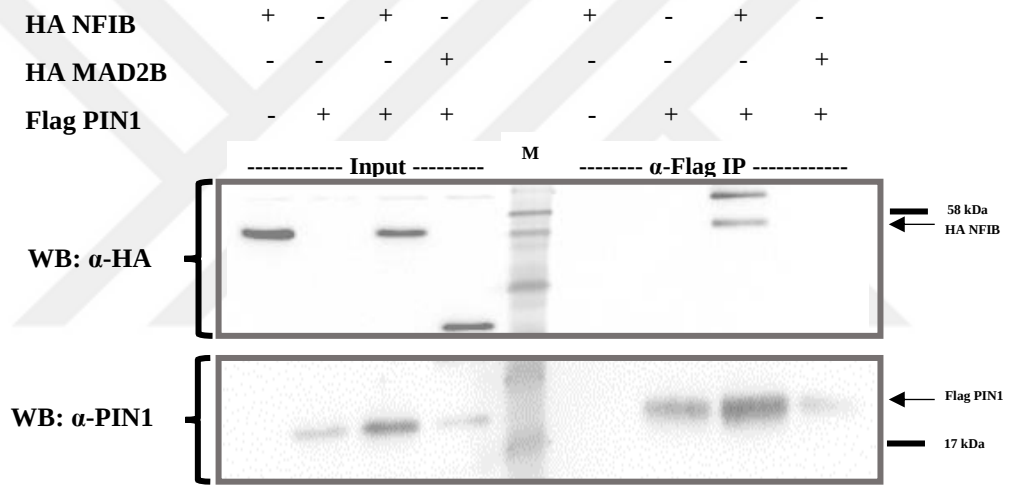

Figure 3.5 : Co-immunoprecipitation analysis of of HA-tagged NFIB and Flag-tagged PIN1. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of Flag-tagged PIN1 using α-Flag antibody followed by western blot analysis of HA-tagged NFIB and HA-tagged MAD2B. Immunoprecipitates are shown together with 10 µg of each input lysate. The position of NFIB and PIN1 immunoreactive bands are indicated with arrows. M: Marker.

Co-immuoprecipitation analysis was extended to other NFI family members. When Flag-tagged PIN1 was co-expressed with each HA-tagged NFI family members in HEK293T cells, yet again HA-tagged NFIB could not be immunoprecipitated with Flag-resin in the absence of Flag-tagged PIN1 (Figure 3.6, lane 6). Moreover, immunoprecipitation of Flag-tagged PIN1 alone did not produce any band on the α-HA blot (Figure 3.7, lane 4). On the other hand, each member of the NFI family could

be co-immunoprecipitated specifically with Flag-tagged PIN1, when they were overexpressed in HEK293T cells (Figure 3.6, Figure 3.7).

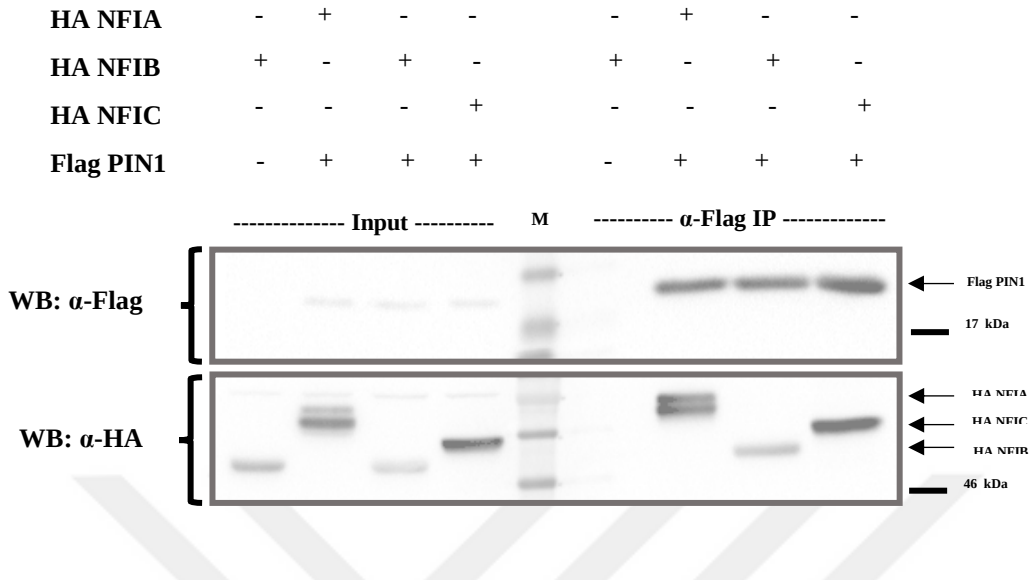

Figure 3.6 : Co-immunoprecipitation analysis of HA-tagged NFIA, NFIB, NFIC and Flag-tagged PIN1. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation of Flag-tagged PIN1 using α-Flag antibody followed by western blot analysis of HA-tagged NFIs. Immunoprecipitates are shown together with 20 µg of each input lysate. The positions of HA immunoreactive bands corresponding to NFIs are indicated with arrows. M: Marker.

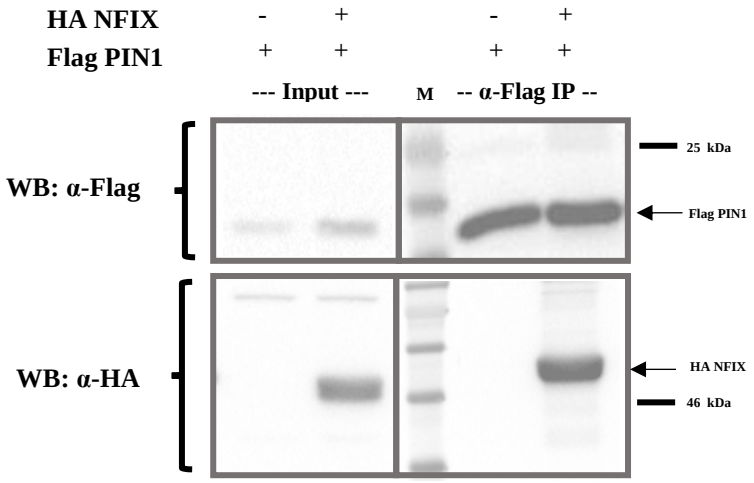

Figure 3.7 : Co-immunoprecipitation analysis of HA-tagged NFIX and Flag-tagged PIN1. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation of Flag-tagged PIN1 using α-Flag antibody followed by western blot analysis of HA-tagged NFIX. Immunoprecipitates are shown together with 20 µg of each input lysate. The position of NFIX immunoreactive band is indicated with arrow. Black line between input lysates and immunoprecipitates indicates that samples run on the same gel but extra lanes were removed. M: Marker.

Next, we performed reciprocal co-immunoprecipitation assays with Flag-tagged PIN1 and HA-tagged NFIA, NFIB, NFIC or NFIX. When Flag-tagged PIN1 was overexpressed alone, little Flag-immunoreactive protein was precipitated with α-HA and when HA-tagged NFIB was overexpressed alone, again small amount of HA-immunoreactive protein precipitated with α-Flag conjugated agarose resin, indicating low levels of overexpressed protein non-specifically bound to resin or antibody. However, the most prominent bands corresponding to co-precipitated proteins were observed in the presence of putative binding partners. Co-immunoprecipitation assay shows that each member of NFI could co-precipitate with PIN1 (Figure 3.8).

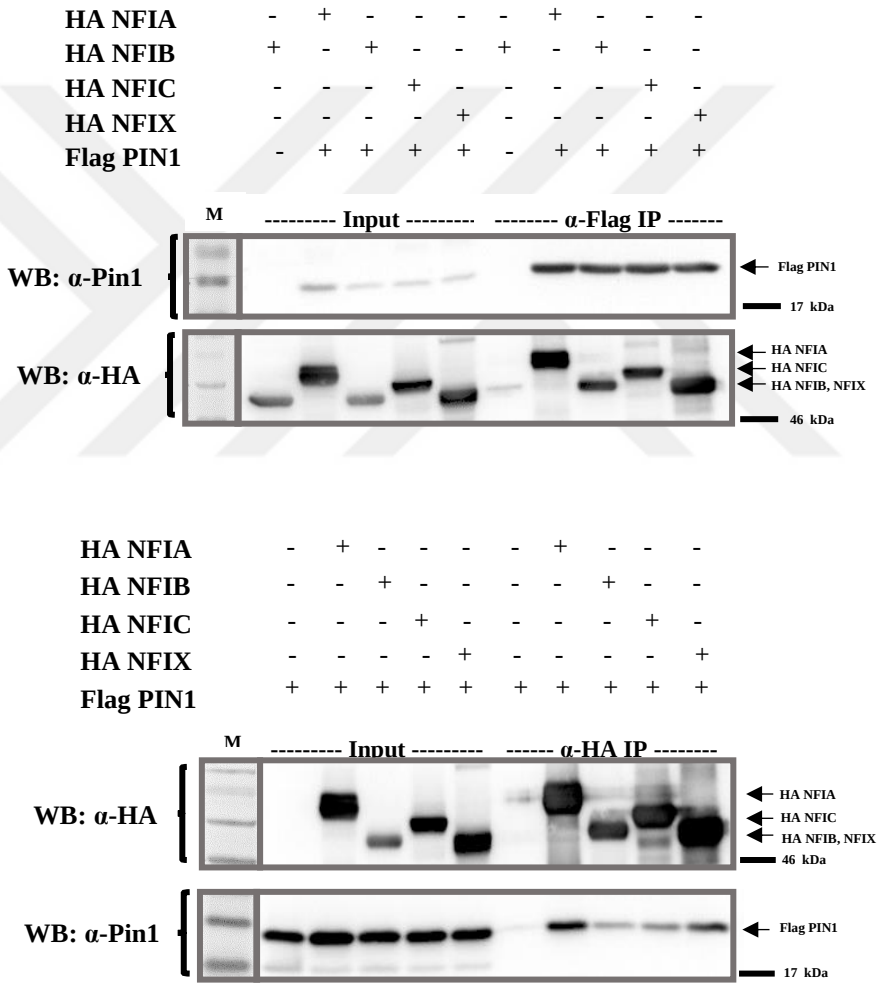

Figure 3.8 : Reciprocal co-immunoprecipitation analyses of HA-tagged NFIs and Flag-tagged PIN1. Membrane extracts from HEK293T cells (0.5 mg) were subjected to immunoprecipitation of Flag-tagged PIN1 using α-Flag antibody and HA-tagged NFIs using α-HA antibody, then followed by western blot analysis of HA-tagged NFIs, and Flag-tagged PIN1, respectively. Immunoprecipitates are shown together with 20 µg of each input lysate. The position of immunoreactive bands are indicated with arrows. M: Marker.

3.3 Analysis of NFI and PIN1 Interactions by GST-Pull Down Assays

We also tested for NFIs and PIN1 interaction using GST pull down experiments. Expression of both Glutathione S transferase (GST) and GST-PIN1 was induced in BL21 *E. coli*, a strain suitable for high-level protein expression of transformed gene under the control of T7 promoter. First we checked for expression of GST-PIN1 fusion protein after one hour of induction. Both GST and GST tagged PIN1 were seen in the soluble fraction of BL21 cell lysates (Figure 3.9).

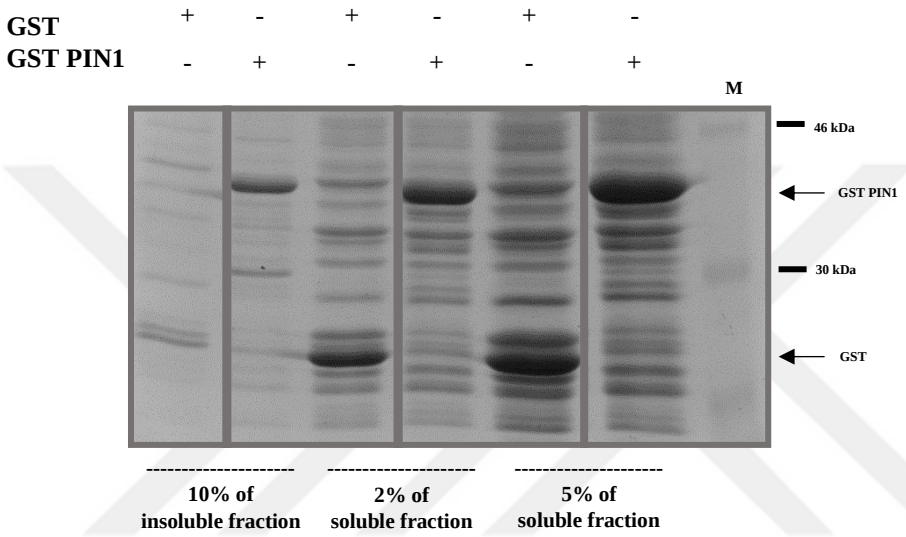

Figure 3.9 : GST-PIN1 is in the soluble fraction of BL21 cells. BL21 cells were transformed with plasmids expressing GST or GST-PIN1, induced with IPTG for 1 hr and lysed using BugBuster. Insoluble fractions were resuspended with 1X Sample Buffer. 10% of insoluble fractions, 2% and 5% of soluble fractions were resolved by 12% SDS-PAGE. Protein bands were visualized by coomassie blue staining. To avoid possible spill over between lanes, gel electrophoresis was performed leaving empty lanes between samples. These lanes were then removed, discontinuous lanes are indicated by boxes. Bands correspond to GST and GST-PIN1 are indicated with arrows. M: Marker.

Solubilized GST and GST-PIN1 were then immobilized on Glutathione Sepharose resin, and allowed to bind individually overexpressed His-tagged NFIB, NFIC and NFIX and HA-tagged NFIA in HEK293T cells. While equivalent amounts of GST and GST-PIN1 precipitated with glutatahione resin, GST-PIN1 pulled down each NFI, whereas GST failed to bind any of them as strongly (Figure 3.10, Figure 3.11 and Figure 3.22).

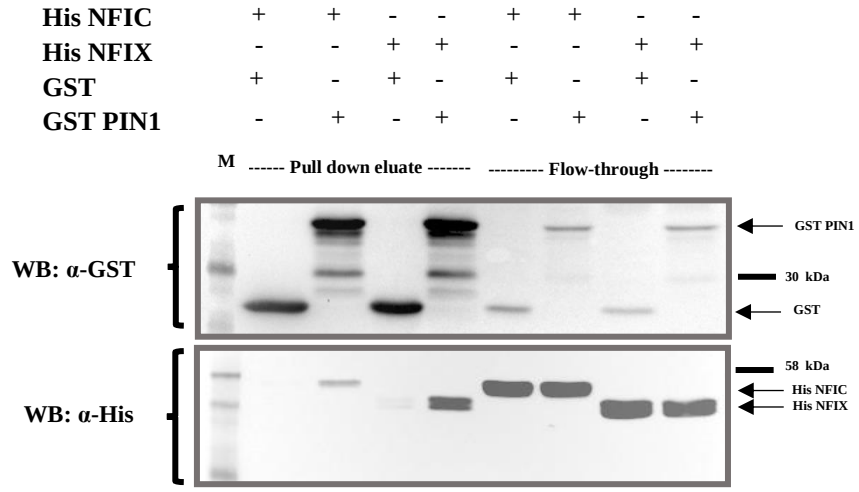

Figure 3.10 : GST Pull down analysis of His-tagged NFIC and His-tagged NFIX with GST-PIN1. 400 µg HEK293T lysate overexpressing His-tagged NFIC or His-tagged NFIX was incubated with bacterially expressed GST or GST-PIN1 immobilized on glutathione sepharose resin. After centrifugation, pellets were eluted in 1X Sample Buffer. 10% of supernatant were loaded as flow-through. Eluates of His-tagged NFIC and NFIX incubated with GST (lane 2, lane 4), His-tagged NFIC and NFIX incubated with GST-PIN1 (lane 3, lane 5), flow through of NFIC incubated with GST and GST-PIN1 (lane 6, lane 7), NFIX incubated with GST and GST-PIN1 (lane 8, lane 9) are shown. Immunoreactive bands correspond to His tagged NFIC, NFIX, GST and GST-PIN1 are indicated with arrows. M: Marker.

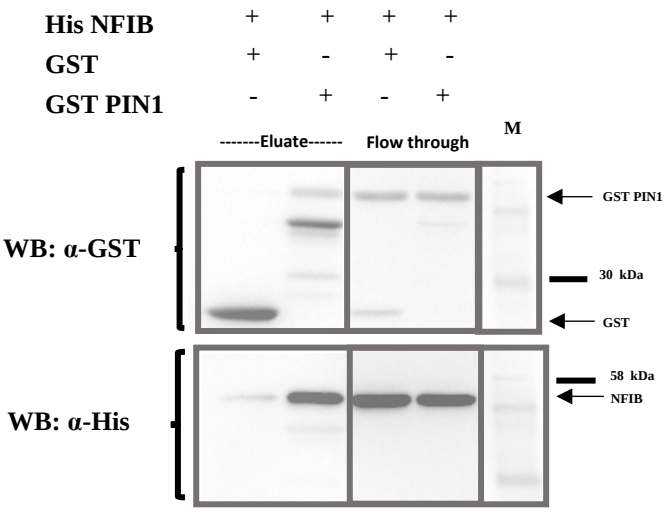

Figure 3.11 : GST Pull down analysis of His-tagged NFIB with GST-PIN1. 150 µg HEK293T lysate overexpressing His-tagged NFIB was incubated with bacterially expressed GST and GST-PIN1 immobilized on glutathione sepharose resin. After centrifugation, pellets resuspenden with 1X Sample Buffer. 10% of supernatant were loaded as flow-through. Eluates of His-tagged NFIB incubated with GST (lane 1), eluates of His-tagged NFIB incubated with GST-tagged PIN1 (lane 2), flow through of NFIB incubated with GST and GST-PIN1 (lane 3, lane 4) are shown. Bands correspond to His tagged NFIB, GST and GST-PIN1 are indicated with arrows. M: Marker.

In order to compare binding affinity of NFIs to PIN1, we first checked for expression levels of plasmids so that lysates with similar amount of overexpressed protein could be used in pull-down experiments. Interstingly, while all HA tagged NFI members are cloned into the same expression vector, expression levels of NFIs differ. Lysates containing approximately equal amounts of overexpressed protein were incubated with either GST or GST-PIN1 (Figure 3.13 left panel). As shown in Figure 3.12, whereas each NFI could be pulled down by GST-PIN1, GST failed to bind any NFI as strongly. In this experiment, PIN1 appeared to interact with NFIB most strongly, with NFIA and NFIC moderately, and with NFIX most weakly.

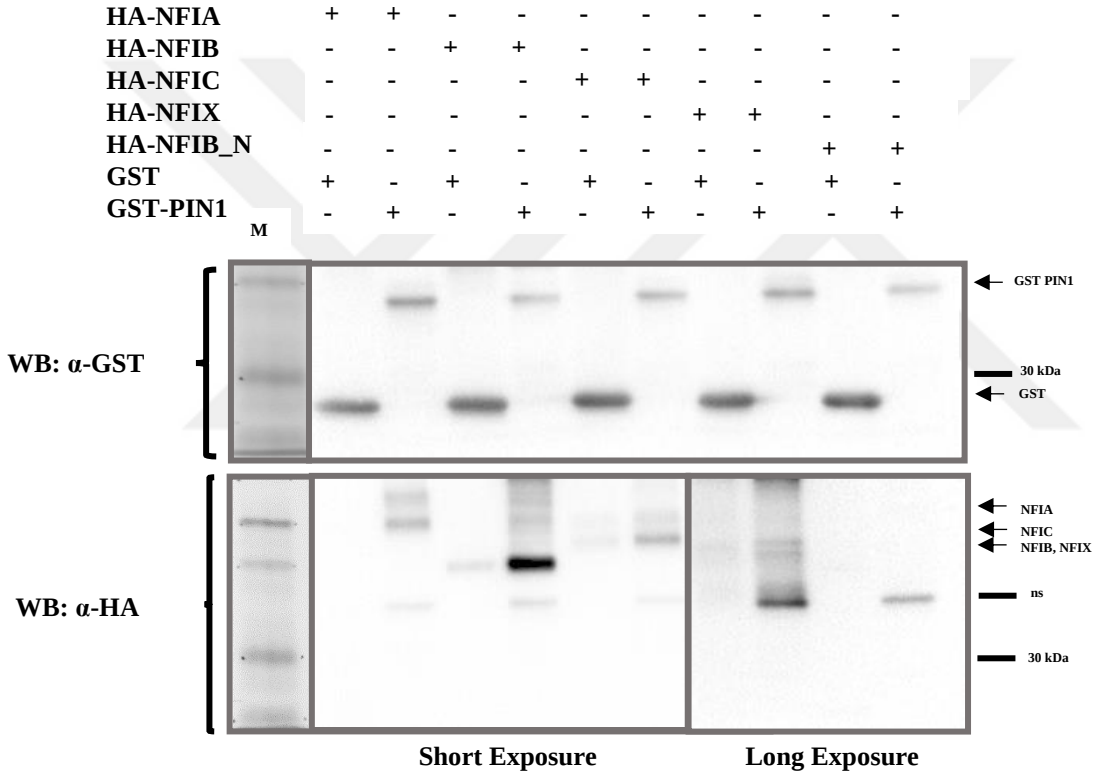

Figure 3.12 : GST pull down analysis of interaction between HA-tagged NFIs and GST-PIN1. 150 µg, 300 µg, 75 µg and 50 µg of NFIA, NFIB, NFIC, NFIX and 100 µg of NFIB_N overexpressed in HEK293T cell lysates were used for pull down with GST-PIN1. 10 µg GST-PIN1 and 15 µg GST were immobilized on resin. Black lines indicate samples run on the same gel but the order of the marker lane was changed. M: Marker. Ns: non-specific.

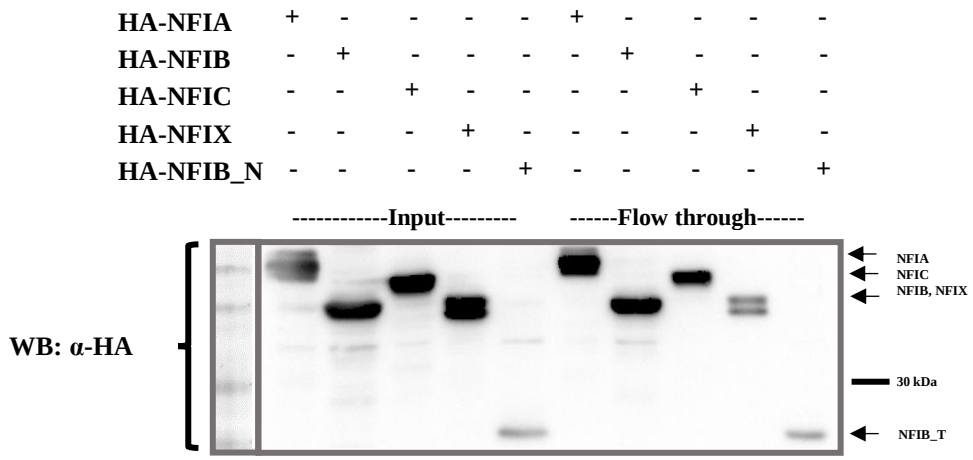

Figure 3.13 : Input lysates of NFIs used in pull down expriment. 20% of input lyates and 10% of flow through are shown on left and right panels, respectively. Immunoreactive bands of HA-tagged NFIs are indicted with arrows. Black line between marker and input indicates that samples run on the same gel but lane order of marker was changed. M: Marker.

These GST pull down experiments showed that each NFI member could be pulled down by GST-PIN1. Overall, these data suggest that PIN1 could interact with each member of NFIs *in vitro*.

3.4 Analysis of NFI Domains Mediating PIN1 Interactions

The finding that PIN1 interacts with each NFI suggested that the N terminal domain, which is conserved among NFIs may be involved in this interaction. In order to investigate which domain of NFIs is required for PIN1 binding, a truncated version of NFIB, which lacks the transactivation or repression domain (HA-tagged N terminal domain of NFIB (HA-NFIB_N) was used in both co-immunoprecipitation and GST pull down experiments. However, expression of HA-NFIB_N in HEK293T cells was significantly lower than the other HA-tagged proteins, especially when co-expressed with other exogenous proteins (Figure 3.12, Figure 3.15). Therefore, in addition to HA-NFIB_N, we used HA-tagged Engrailed NFIB_N fusion protein, a dominant negative NFI, which inhibits transactivation by all NFI when co-expressed along with these proteins (Bachurski et al., 2003) and as it contains the NFIB N-terminal domain, provides another construct to test for N-terminal domain and PIN1 interactions. Structures of proteins used in co-immunoprecipitation and pull down experiments are shown in Figure 3.14.

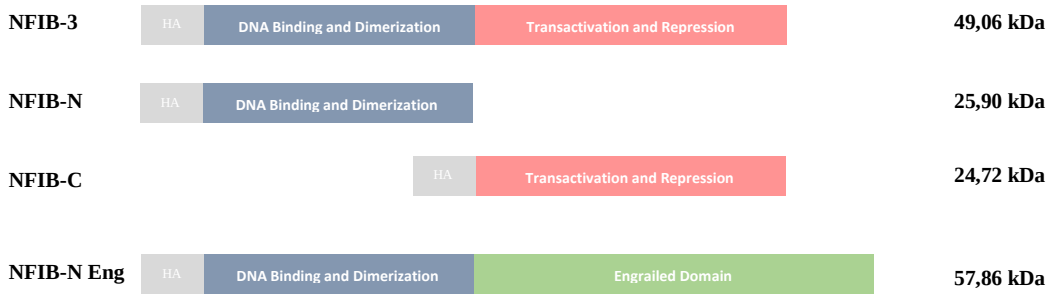

Figure 3.14 : Structures of NFIB constructs used in analyses. NFIB-3 isoform has a DNA binding and dimerization domain and a transactivation and repression domain. NFIB-N comprises the N terminal domain of NFIB or the DNA binding and dimerization domain. NFIB-C comprises the C terminal domain of NFIB or the transactivation and repression domain. NFIB-N Eng contains the N terminal DNA binding domain of NFIB fused to the Engrailed repression domain. Each construct was expressed with an HA-tag located at their N terminus.

When expressed in HEK293T cells, a small amount of Engrailed NFIB_N is immunoprecipitated with α-Flag indicating that N terminal domain of NFIB may be responsible for interaction with PIN1 (see Figure 3.15, lane 9). However, as both NFIB_N or Engrailed NFIB_N are expressed at very low levels, these experiments were difficult to interpret (Figure 3.15).

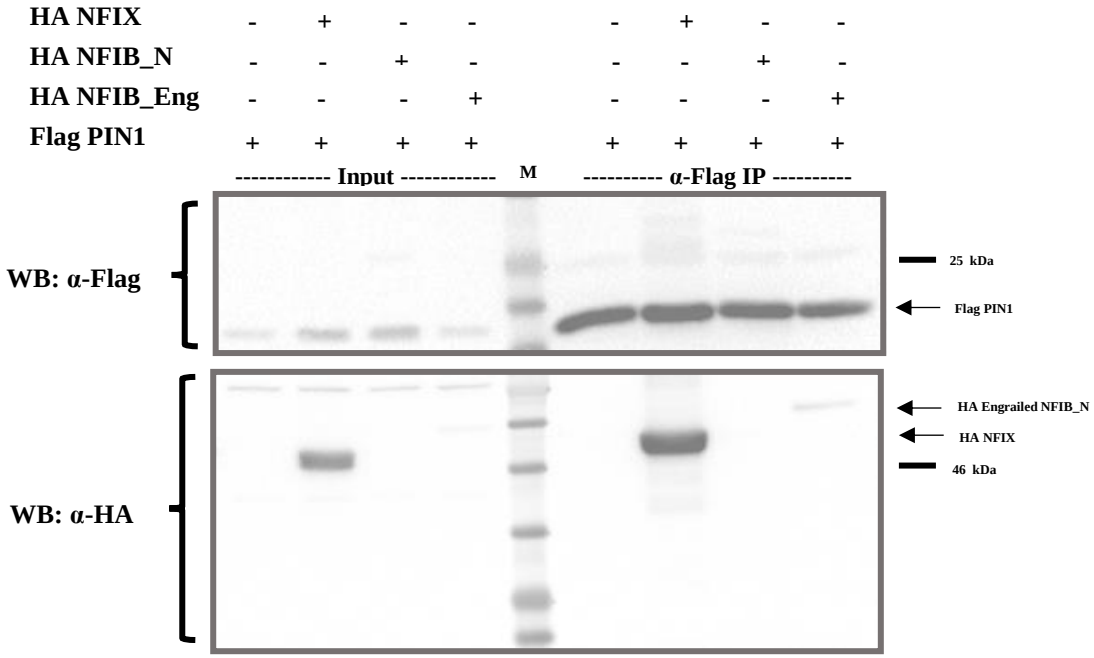

Figure 3.15 : Co-immunoprecipitation analysis of HA-tagged NFIB_N, HA-tagged NFIB_N_Eng and Flag-tagged PIN1.

Figure 3.15 Cont. : Co-immunoprecipitation analysis of HA-tagged NFIB_N, HA-tagged NFIB_N_Eng and Flag-tagged PIN1. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation using α-Flag antibody. Immunoprecipitates were loaded together with 20 µg of each input lysate. The porsitions of immunoreactive bands of NFIX and NFI_N_Eng were indicated with arows.. M: Marker.

We also performed, GST pulldown assays with HA-tagged NFIB_N overexpressing HEK293T lysates. No bands were observed in pull down lanes that correspond to NFIB_N (Figure 3.13). Pull down experiment was repeated to assess binding of HA-tagged NFIB_N to PIN1, using HA-tagged NFIB as positive control for interaction with PIN1 and HA-tagged LDB1 as a putative negative control, to eliminate false positive interactors. Data indicates that NFIB could be pulled down by GST-tagged PIN1 while neither HA-tagged LDB1 nor NFIB_N were pulled down by PIN1 (Figure 3.16).

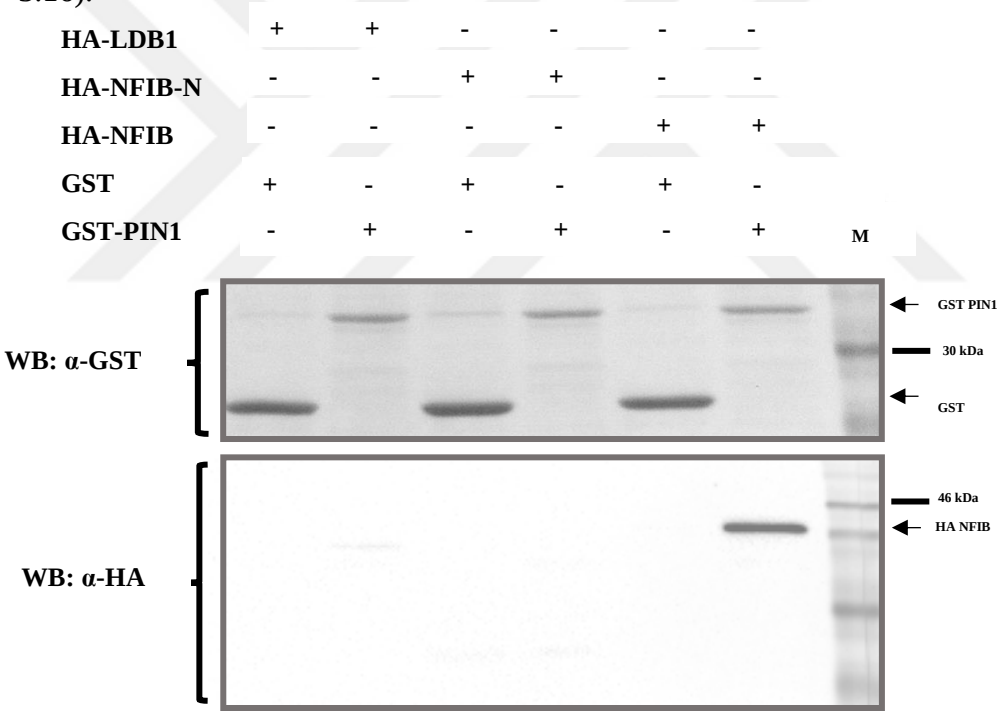

Figure 3.16 : GST pull down analysis of HA-tagged NFIB_N and GST-tagged PIN1 interaction. 90 µg of HEK293T cell lysates overexpressing LDB1, Equal amounts of GST and GST-PIN1 were used in pull down assay (Upper panel). NFIB, and NFIB_N were used for pull down with GST-tagged PIN1 (Lower panel). M: Marker.

These results suggest that either N terminus of NFIB is not involved in NFI interactions with PIN1 or any interaction is not detected because of low expression levels of that protein. As initial data suggested that N-terminal domain is not involved, we searched

for PIN1 recognition motifs within the diverse C-terminal domains of NFIs and found several conserved Ser-Pro PIN binding motifs that may be required for interaction with PIN1. To investigate NFIB C-terminal domain and PIN1 interactions, we used HA-tagged C-terminal domain of NFIB (NFIB_C) in pull down experiments (See Figure 3.14). We also used maximum levels of NFIB_N in these experiments. Data showed that C-terminal domain could not be pulled down by PIN1 (Figure 3.21, Figure 3.23) whereas a faint band was observed for NFIB_N incubated with GST-PIN1 (Figure 3.21). These results suggested that N terminal DNA binding and dimerization domain might be important for interactions with PIN1.

3.5 Mutation Analysis of Potential Residues on NFIB That May Mediate Interaction with PIN1

Both the N-terminal DNA binding and dimerization domain and the C-terminal transactivation and repression domain of NFIs contain several conserved (S/T)P motifs (Figure 3.17), and at least six of them are phosphorylated in cells (Olsen et al., 2006; Dephoure et al., 2008; Olsen et al., 2010).

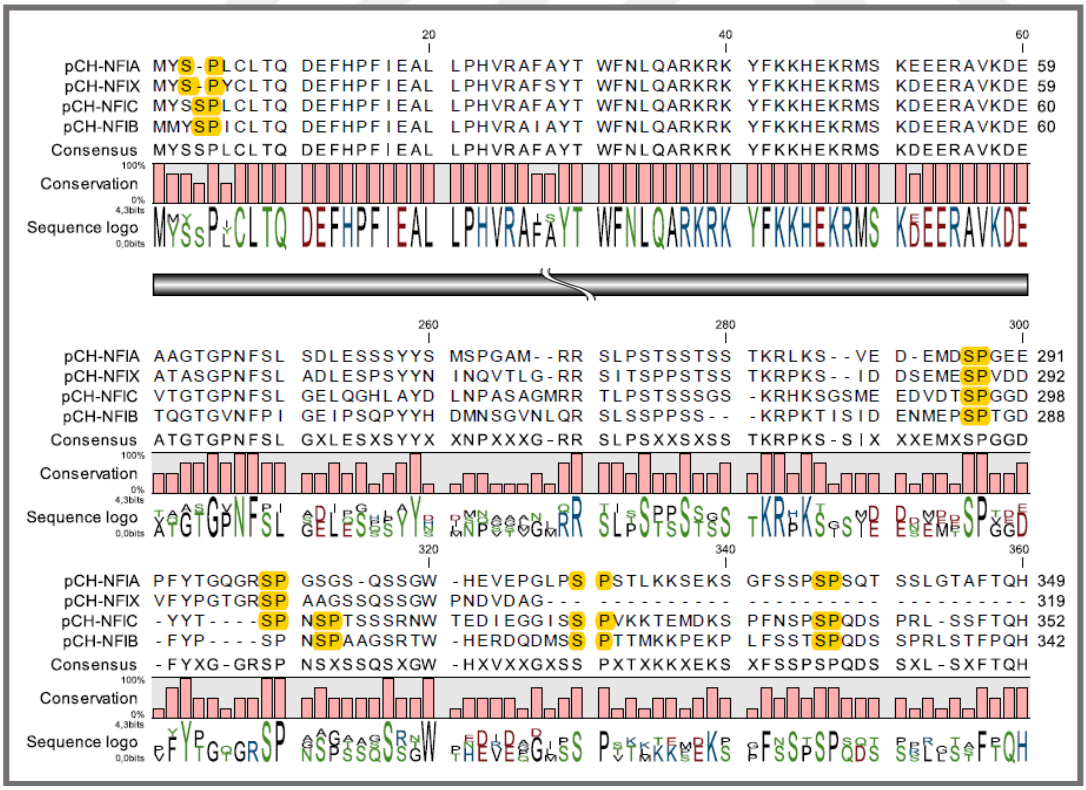

Figure 3.17 : Phosphorylated serine-proline (S-P) residues conserved among NFIs. SP residues, potentially recognized by PIN1, were labeled as yellow. Sequences were aligned using CLC Main Workbench 7 software. These residues have been reported to be phosphorylated.

To determine whether interaction between PIN1 and NFIs depends on phosphorylation of these conserved SPs, we created NFIB mutants where a single or multiple serines are substituted to alanine in the C-terminal domain. Clones carrying the single mutation S284A, triple mutation S292A, S295A, S312A and quadruple mutation S292A, S295A, S312A, S328A were generated and will be referred to as NFIB SA_1, NFIB SA_3, NFIB SA_4 according to the total number of mutated serine residues. GST pull-down and co-immunoprecipitation experiments were performed to investigate interactions between mutant and wild-type NFIB and PIN1. HA-tagged wild type or mutant NFIBs were overexpressed in HEK293T cells and incubated with GST-PIN1 or GST alone. Pull-down experiments showed that mutant NFIBs could still interact with GST-PIN1 (Figure 3.18, Figure 3.19).

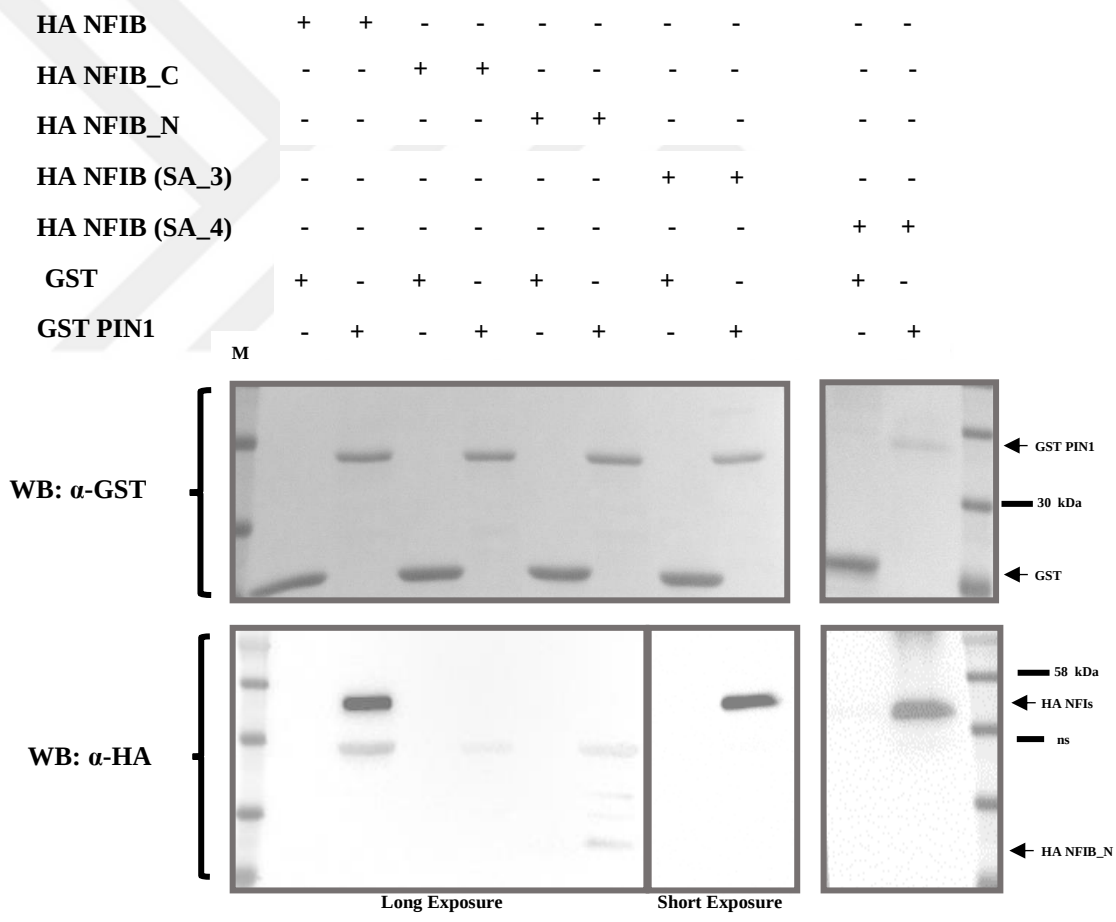

Figure 3.18 : Analysis of interaction between mutated NFIB and PIN1 via GST pull down. 100 µg HEK293T cell lysates including overexpressed wild-type or mutated NFIB proteins were incubated with GST-PIN1 or GST alone, previously immobilized on glutathione resin. The positions of the immunoreactive bands are indicated with arrows. Black line between sample indicates that images of different exposure time were used. M: Marker. Ns: non-specific.

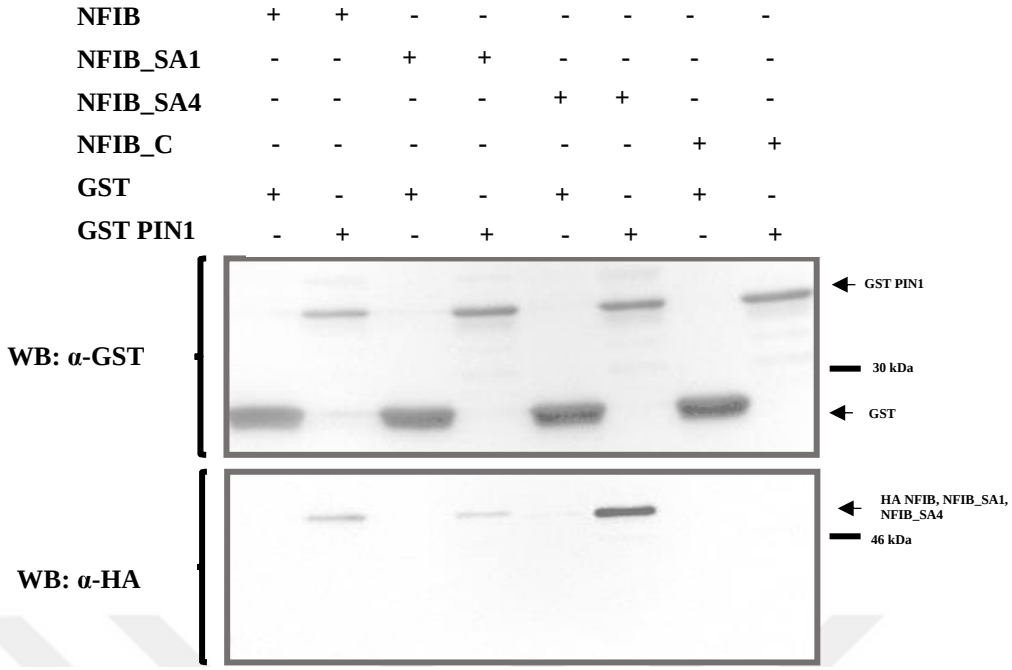

Figure 3.19 : GST pull down analysis of NFIB_SA1, NFIB_SA4,NFIB_C and PIN1 interactions. Eluates of mutated NFIBs and NFIB_C incubated with GST (lane 1, 3, 5, 7). Eluates of mutated NFIBs and NFIB_C incubated with GST-PIN1 (lane 2, 4, 6, 8). The positions of immunoreactive bands are indicated with arrows.

In addition, we co-expressed HA-tagged mutated NFIBs along with Flag-tagged PIN1 and performed co-immunoprecipitations with α-HA and α-Flag conjugated agarose resin. Consistent with the pull down experiment, mutated NFIs and PIN1 co-immunoprecipitated with each other (Figure 3.20, Figure 3.21). Taken together these results indicate that - S284, S292, S295, S312, S328 in NFIB may not be involved in interactions with PIN1.

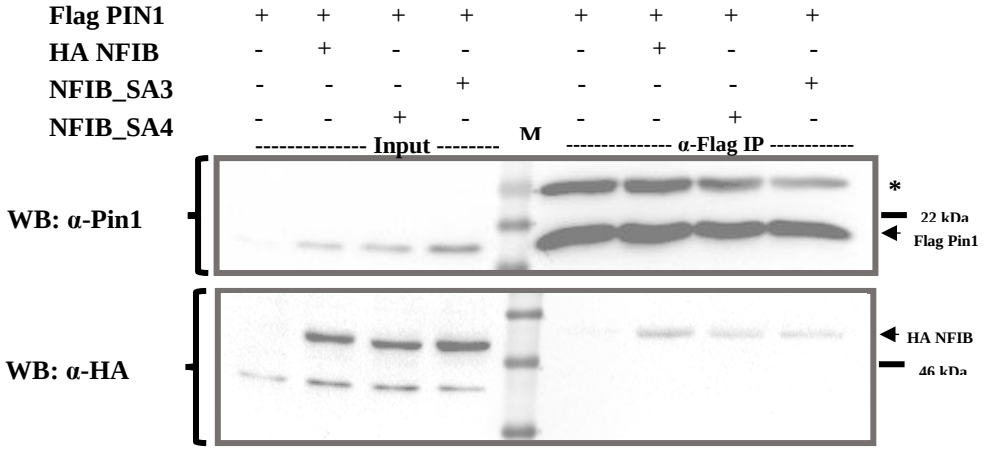

Figure 3.20 : Co-immunopreipitation analysis of mutated NFIBs and PIN1 interactions.

Figure 3.20 (Cont.) : Co-immunopreipitation analysis of mutated NFIBs and PIN1 interactions. 0.5 mg HEK293T cell lysates including HA-tagged NFIB, NFIB_SA4 or NFIB_SA3 overexpressed along with Flag-tagged PIN1 were immunoprecipitated using α-Flag crosslinked agarose resin. Immunoprecipiates are shown together with 20 μg of input lysate. Positions of immunreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

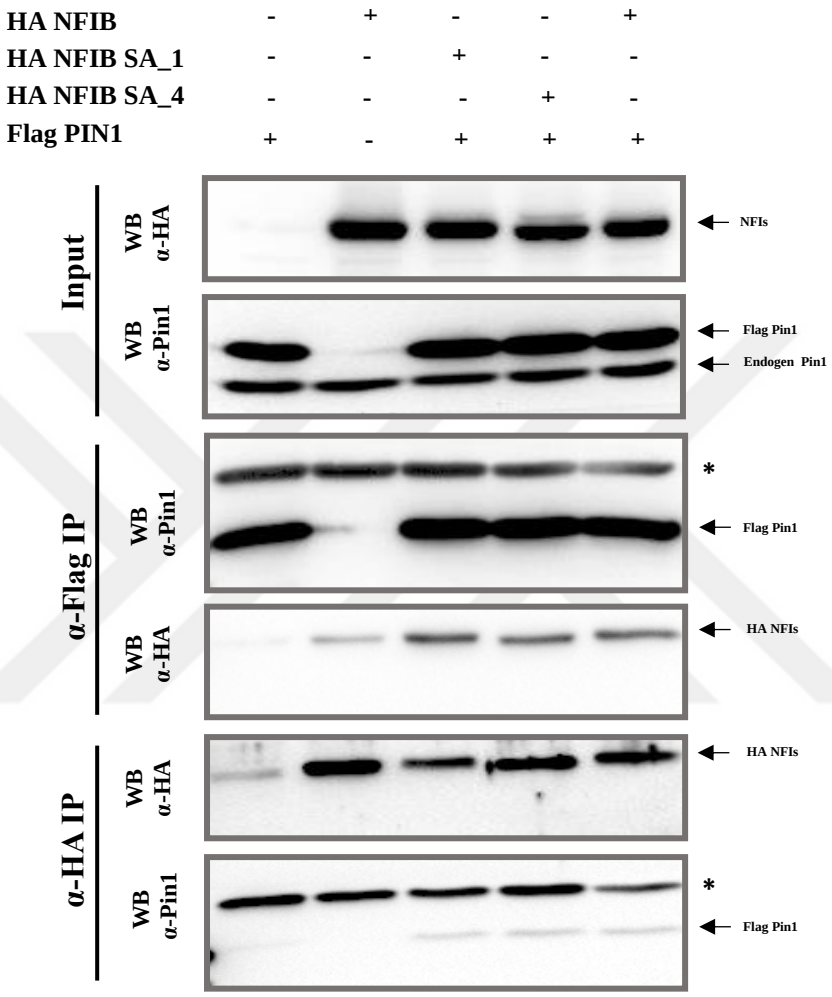

Figure 3.21 : Co-immunoprecipitation analysis of HA tagged mutated NFIBs and Flag-tagged PIN1. 0.5 mg HEK293T cell lysate overexpressing Flag-tagged PIN1 and HA-tagged NFIs. α-HA and α-Flag conjugated agarose resin. were used for immunoprecipitation of HA-tagged and Flag-tagged proteins. Immunoprecipitates are shown with 40 μg input lysate. The positions of immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

3.6 Phosphatase Treatment Did Not Inhibit NFI and PIN1 Interactions

In order to understand whether interaction of NFIs and PIN1 is dependent on phosphorylation of NFIs, calf alkaline phosphatase (CIP) was used to remove phosphate groups of proteins. We treated cell lysates obtained from HEK293T cells

with CIP before incubation with GST or GST-PIN1 immobilized on glutathione sepharose, to test if any phosphorylated residues are required for interaction with NFIs. Additionally, as an alternative procedure, NFIs and GST-PIN1 complexes on glutathione sepharose resin was treated with CIP before elution to test if any phosphorylated residues are required once the proteins are bound to each other. As shown in Figure 3.22, treatment of glutathione sepharose resin incubated with overexpressed NFIA or pretreatment of that lysate with CIP did not lead to dissociation of NFIA from PIN1. Treatment of glutathione sepharose resin incubated with overexpressed NFIB and NFIX with CIP again had no effect on PIN1 interactions with NFIB or NFIC (Figure 3.23, Figure 3.24). NFIA, NFIB, or NFIC and GST tagged PIN1, NFIs and PIN1 still bound to each other under CIP treated conditions. These results may indicate that interaction between PIN1 and NFIs may be maintained irrespective of CIP treatment or interaction may occur phosphorylation independent manner, however as we did not include any positive controls, these findings remain inconclusive.

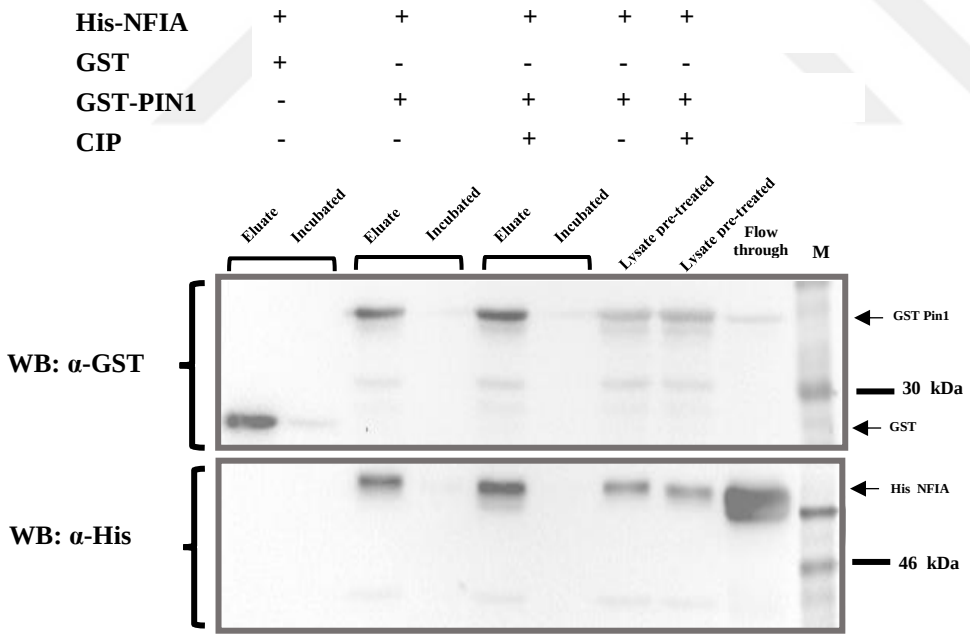

Figure 3.22 : Analysis of NFIA and PIN1 interaction after CIP treatment. 100 µg His tagged NFIA was incubated with GST or GST tagged PIN1 immobilized on resin. CIP (20 units) treatment was performed on resin (lane 1-6), or lyste (lane 7-8). CIP pretreated sample was loaded as flow through (lane 9). The positions of immunorective bands are indicated with arrows. M: Marker.

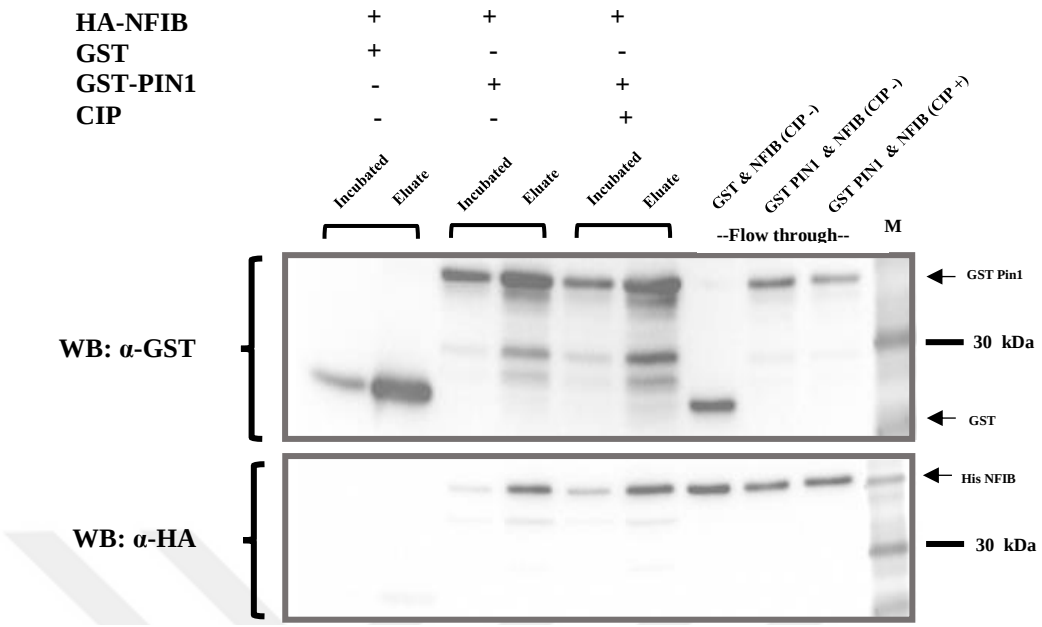

Figure 3.23 : Analysis of NFIB and PIN1 interaction after CIP treatment. 100 µg HA tagged NFIB was incubated with GST and GST-PIN1 immobilized on resin. CIP treatment was performed on glutathione sepharose resin. NFIB incubated with GST (lane 1, 2) and GST-PIN1 (lane 3-6) and 10% of flow through was loaded for indicated samples (lane7-9). The position of immunoreactive bands are shown with arrows. M: Marker.

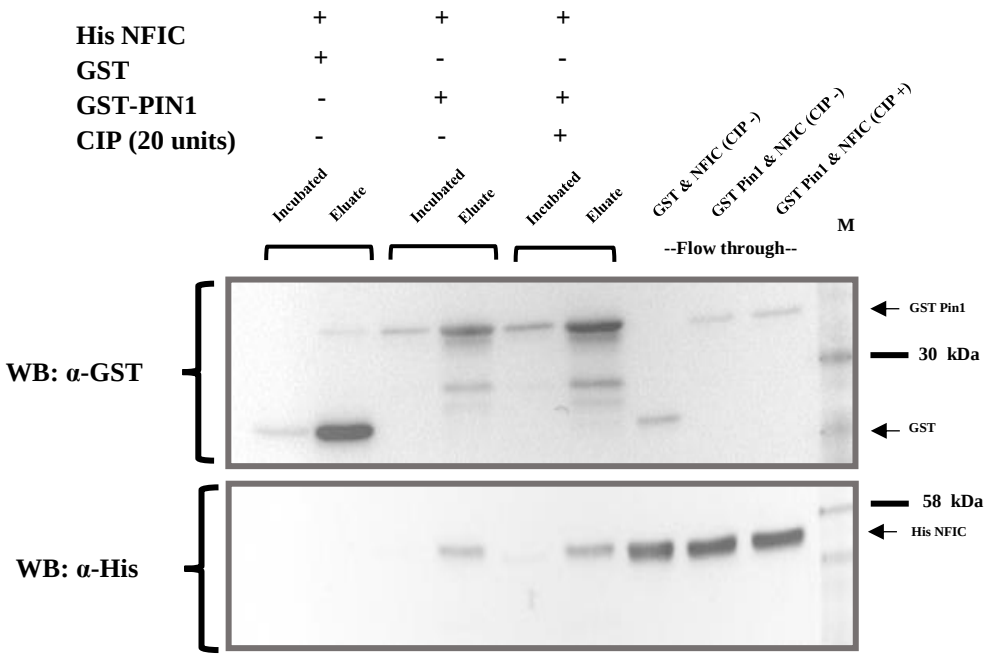

Figure 3.24 : Analysis of NFIC and PIN1 interaction after CIP treatment. 100 µg His tagged NFIC was incubated with GST (lane 1-2) and GST-PIN1 (lane 3-6) immobilized on resin. CIP treatment was performed on glutathione sepharose resin. 10% of flow through was loaded for indicated sample. The positions of immunoreactive bands are shown with arrows. M: Marker.

3.7 Analysis of NFI and LMO4 Interactions by Co-Immunoprecipitation Assays

Next, we focused on LMO4 and its interactions with NFIs. To this end, HA-tagged NFIA, NFIB, NFIC and NFIX were overexpressed in HEK293T cells along with Myc-tagged LMO4. To control for non-specific binding, HA-tagged MAD2B was also co-expressed along with Myc-tagged LMO4. Immunoprecipitation of Myc-tagged LMO4 overexpressing HEK293T cell lysates with α-HA conjugated agarose resin did not detect any bands indicating Myc-tagged LMO4 does not bind α-HA conjugated agarose resin non-specifically (Figure 3.25). Co-immunoprecipitation assays performed in two independent experiments using α-HA conjugated agarose resin revealed that Myc-tagged LMO4 could be co-immunoprecipitated with HA-tagged NFIA, NFIC and NFIX whereas co-immunoprecipitation with HA tagged NFIB was not detected (Figure 3.25 and Figure 3.26).

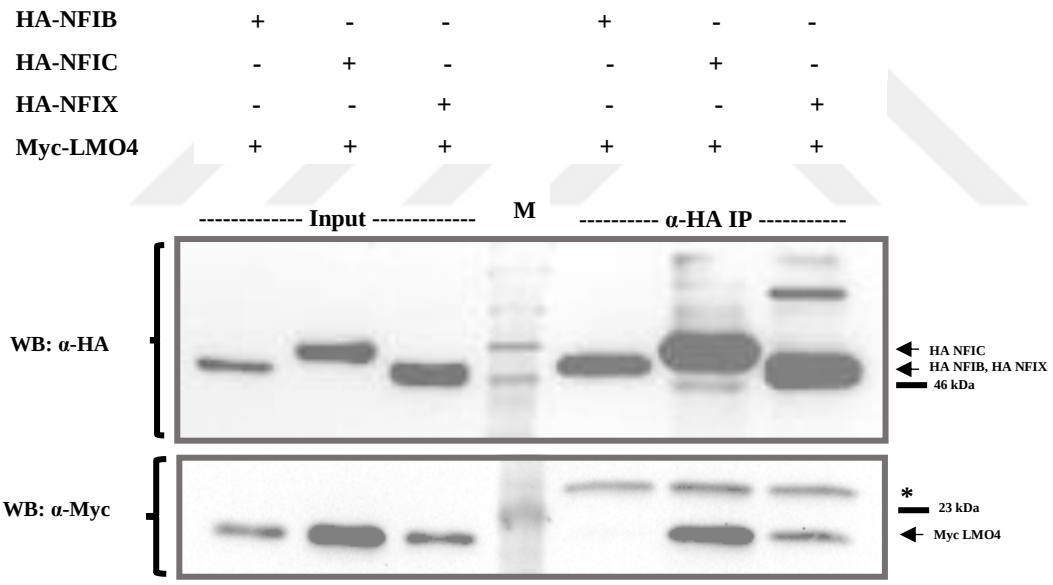

Figure 3.25 : Co-immunoprecipitation analysis of NFIB,NFIC, NFIX and LMO4. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation of HA-tagged NFIs using α-HA antibody followed by western blot analysis of Myc-tagged LMO4. Immunoprecipitates are shown together with 20 μg of each input lysate. The positions of NFIB, NFIC, NFIX and LMO4 immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

Since HA-tagged NFIB binding to with Myc-tagged LMO4 could not be observed in two independent experiments, we used HA-tagged LDB1, previously identified as an interacting partner of LMO4, as a positive control for co-immunoprecipitation

experiments. HA-tagged NFIB_N was used to investigate interaction of LMO4 and N-terminal domain. Additionally, HA-tagged MAD2B was used as a putative negative control to eliminate false positive interactors. We transfected HEK293T cells with plasmids expressing HA-tagged NFIB, HA-tagged NFIB_N, HA-tagged LDB1, or HA tagged MAD2B along with plasmid expressing Myc-tagged LMO4. Immunoprecipitation assay using α-HA crosslinked resin showed that HA-tagged LDB1 could be precipitated with LMO4 as expected, however, HA-tagged NFIB and HA-tagged NFIB_N did not precipitate Myc-tagged LMO4 (Figure 3.26).

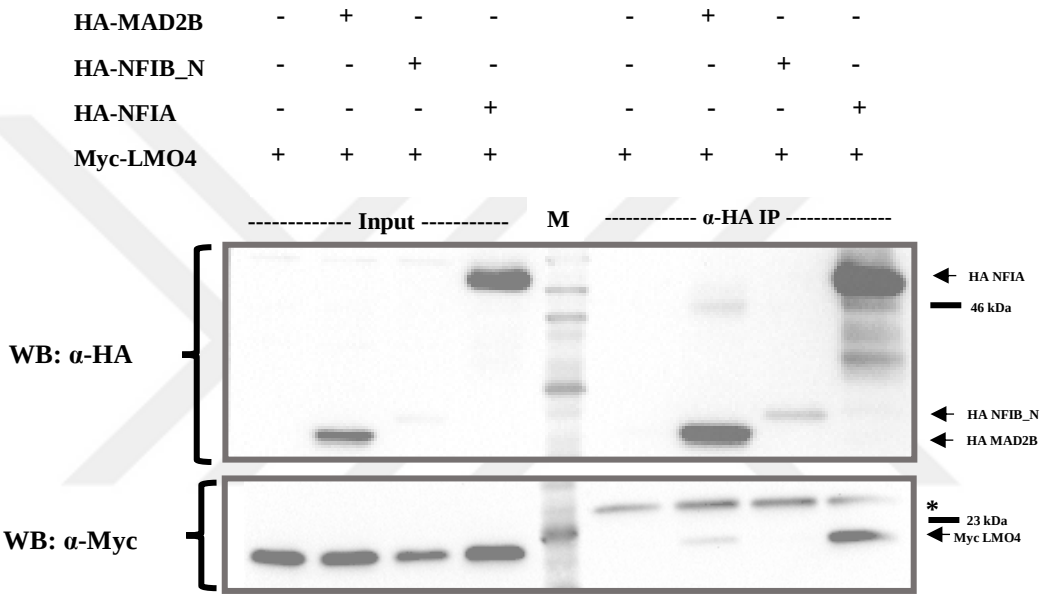

Figure 3.26 : Co-immunoprecipitation analysis of NFIA, MAD2B, NFIB_N and LMO4. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation of HA-tagged NFIA, HA-tagged NFIB_N and HA-tagged MAD2B using α-HA antibody followed by western blot analysis of Myc-tagged LMO4. Immunoprecipitates are shown together with 20 µg of each input lysate. The positions of NFIA, NFIB_N, MAD2B and LMO4 immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

Next we performed the reverse experiment: we immunoprecipitated Myc-tagged LMO4 and assayed for the presence of NFIs. Myc-tagged LMO4 interacted only with HA-tagged NFIX (Figure 3.27; Figure 3.28) Reciprocal co-immunoprecipitation experiment showed that indeed Myc-tagged LMO4 binds HA-tagged NFIX alone (Figure 3.29; Figure 3.30). Immunoprecipitation of Myc-tagged LMO4 did not result in co-precipitation of NFIA, NFIB or NFIC. Moreover, HA-tagged NFIB_N or HA-tagged NFIB_Eng did not interact with LMO4 (Figure 3.28; Figure 3.30). Together

these results indicate that LMO4 could physically interact with NFIX in HEK293T cells when they are overexpressed.

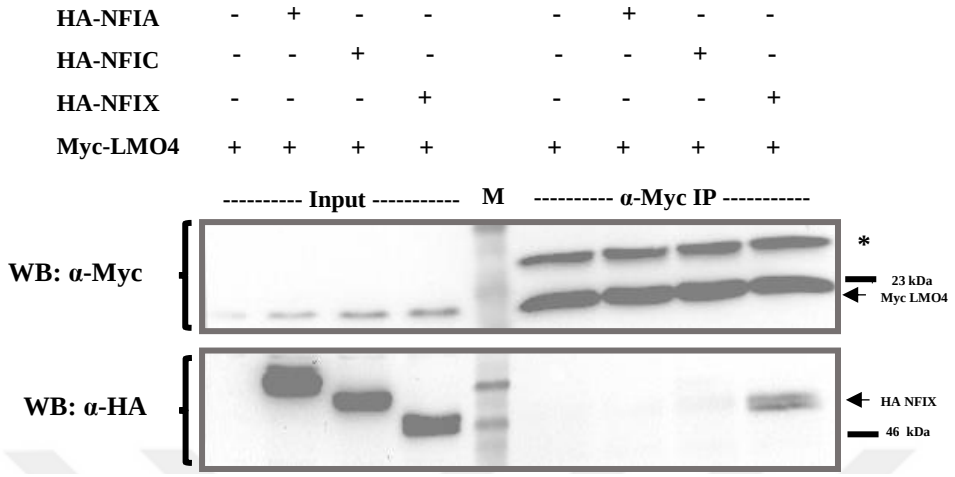

Figure 3.27 : Co-immunoprecipitation analysis of NFIA, NFIC, NFIX and LMO4. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation of Myc-tagged LMO4 using α-Myc antibody followed by western blot analysis of HA-tagged LMO4. Immunoprecipitates are shown together with 20 µg of each input lysate. The positions of immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

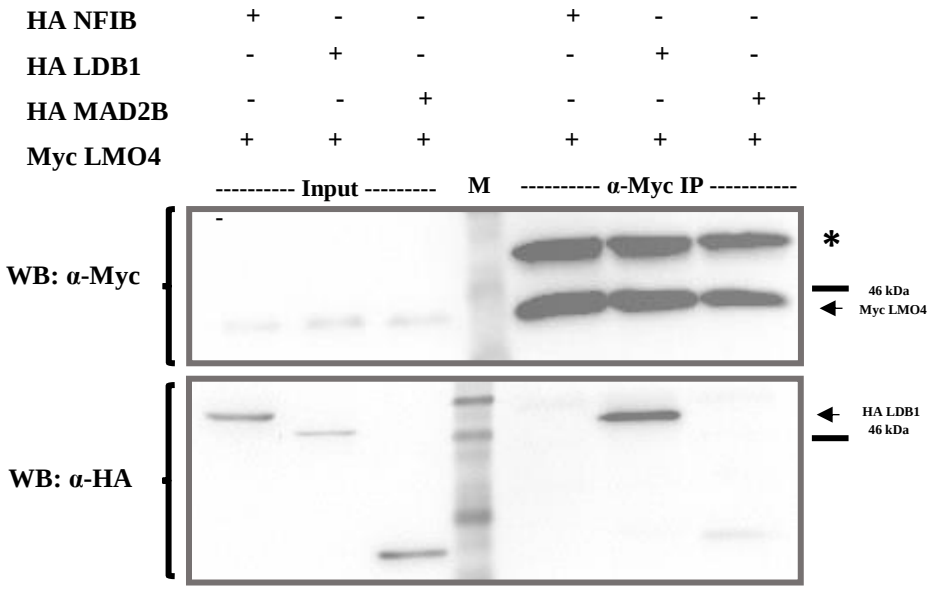

Figure 3.28 : Co-immunoprecipitation analysis of NFIB, MAD2B, LDB1 and LMO4. Membrane extracts from HEK293T cells (1 mg) were subjected to immunoprecipitation of Myc-tagged LMO4 using α-Myc antibody followed by western blot analysis of HA-taggedNFIB, NFIB_N and LDB1. Immunoprecipitates are shown together with 20 µg of each input lysate. The positions of immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

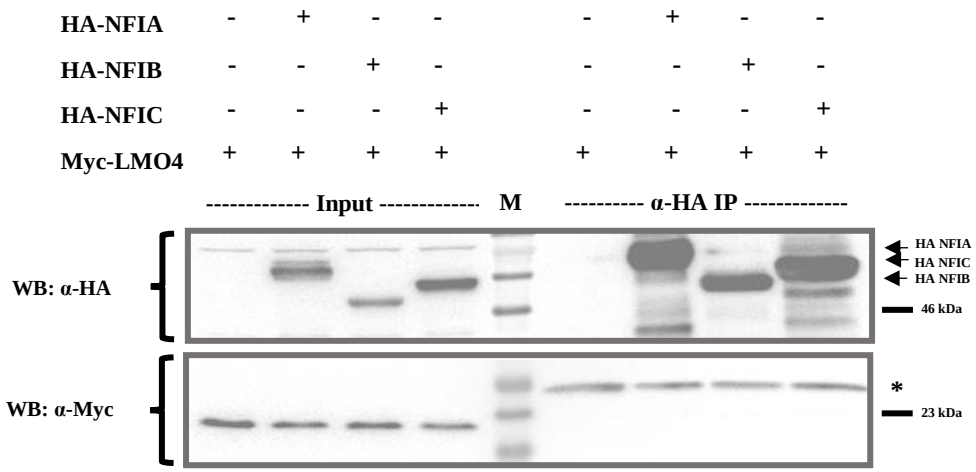

Figure 3.29 : Co-immunoprecipitation analysis of NFIA, NFIB, NFIC and LMO4. Membrane extracts from HEK293T cells (1 mg) were subjeced to immunoprecipittion of HA-tagged NFIs using α-HA antibody followed by western blot analysis of Myc-tagged LMO4. Immunoprecipitates are shown together with 20 µg of each inpt lysate. The position of immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

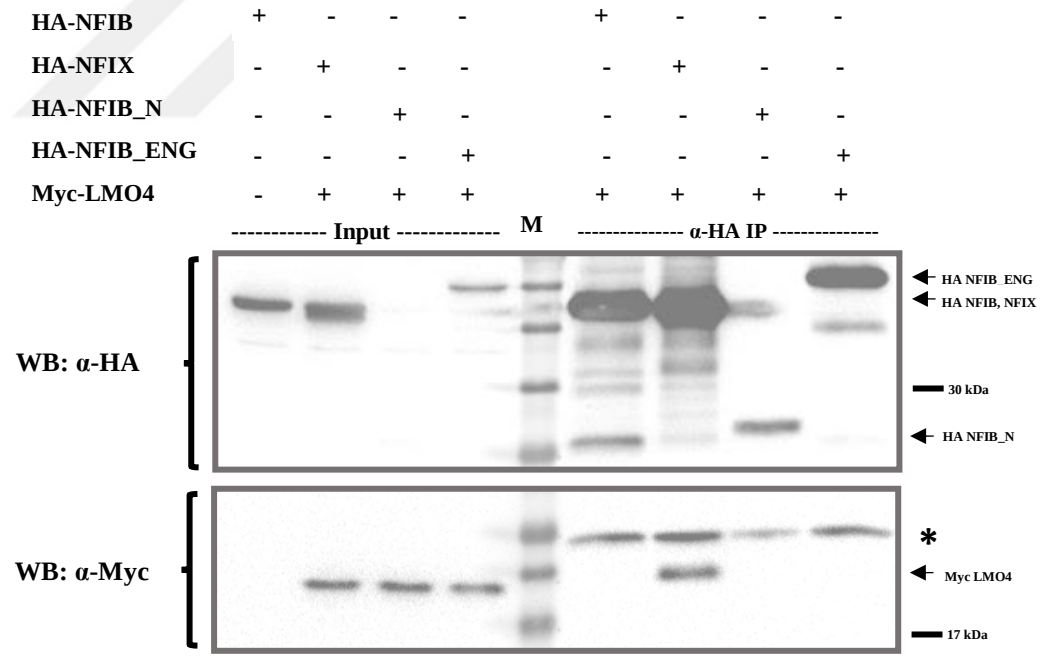

Figure 3.30 : Co-immunoprecipitation analysis of NFIX, NFIB_N, NFIB_Eng and LMO4. Membrane extracts from HEK293T cells (1 mg) were subjeced to immunoprecipittion of HA-tagged NFIs using α-HA antibody followed by western blot analysis of Myc-tagged LMO4. Immunoprecipitates are shown together with 20 µg of each inpt lysate. The position of immunoreactive bands are indicated with arrows. Asterisk indicates IgG light chain. M: Marker.

3.8 Analysis of NFI and LMO4 Interaction by GST-Pull Down Assays

Next we investigated NFIs- LMO4 interactions using GST-pull down assays. GST-LMO4 fusion protein expression was induced in BL21 *E. coli*, and 2hrs after induction, BL21 cells were lysed in BugBuster reagent. However, GST-LMO4 was found to be in the insoluble fraction after cell lysis (Figure 3.31).

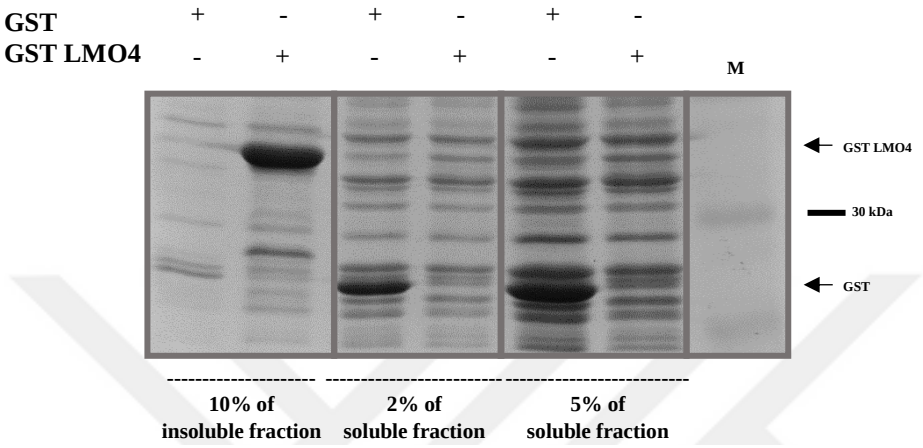

Figure 3.31 : GST-LMO4 is in the insoluble fraction of BL21 cell. BL21 cells were transformed with plasmid expressing GST or GST LMO4 and induced with IPTG for 1 hr. Cells were lysed using BugBuster reagent. Insoluble fraction was diluted with 1X Sample Buffer. 10% of insoluble fractions, 2% and 5% of soluble fractions were resolved by 12% SDS-PAGE. Protein bands were visuailized by coomassie blue staining. To avoid possible spill over between lanes, gel electrophoresis was performed leaving empty lanes between samples. These lanes were then removed, discontinuous lanes are indicated by boxes. Bands coressponding to GST and GST-LMO4 are indicated with arrows. M: Marker.

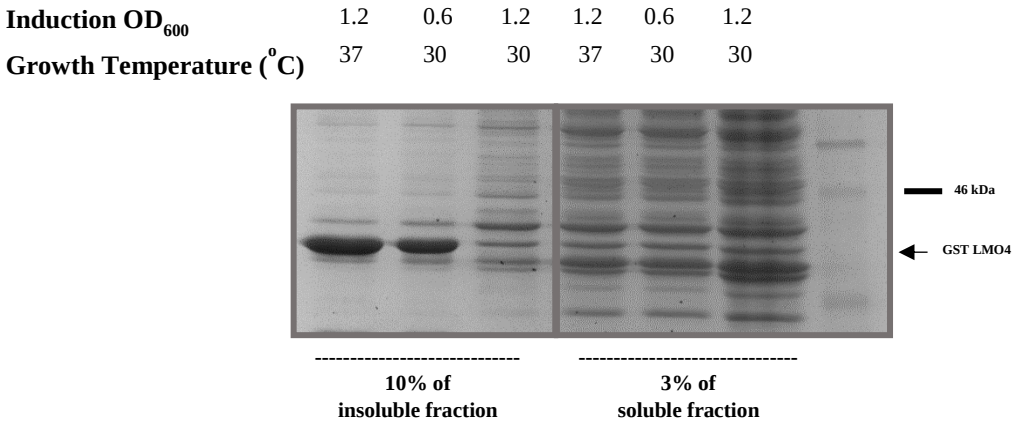

Figure 3.32 : GST-LMO4 is in the insoluble fraction of BL21 cell lysate.

Figure 3.32 Cont. : GST-LMO4 is in the insoluble fraction of BL21 cell lysate. Cells were induced at late OD_{600} or grown at lower temperature after induction and lysed using BugBuster. Pellets were re-suspended with 1X Sample Buffer. 10% of insoluble and 3% of soluble fractions were loaded. Protein bands were visualized by coomassie blue staining. Bands corresponding to GST and GST-LMO4 are indicated with arrows. M: Marker.

To avoid any problems due to incorrect folding or aggregation of overexpressed proteins, BL21 cells were grown at a lower temperature and/or induced later, at higher OD values. As shown in Figure 3.32, growth at lower temperature or induction at higher ODs was not sufficient to recover GST-LMO4 from the insoluble fraction.

There are several studies that report problems with solubilization of LMO4 when expressed in bacteria. It has been shown that fusion with LID domain of LDB1 stabilizes LMO4 structure and facilitates its purification as a soluble protein during isolation (Asper et al., 2011; Deane et al., 2003; Deane et al., 2001). In order to obtain GST- LMO4 in *E. coli* in a soluble and stable form, 38 residues of LID domain of LDB1 were fused using GGSGGHMGSGG linker to the C-terminus of the LMO4 (Figure 33). BL21 cells were transformed with this plasmid expressing GST-LMO4 LID. After 1 hour induction, the fusion protein was obtained in the soluble fraction of *E. coli* cell lysate (Figure 3.34).

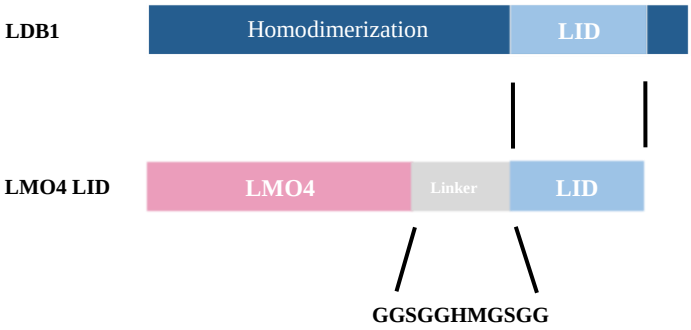

Figure 3.33 : Schematic diagram of LMO4 LID fusion protein. Lim Interactor Domain (LID) of LDB1 was fused to the C-terminal domain of the LMO4.

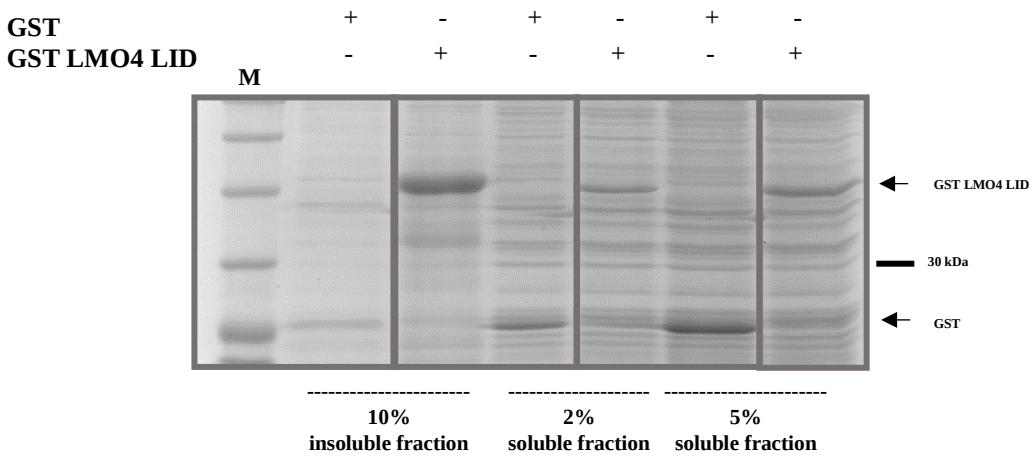

Figure 3.34 : GST-LMO4 LID is in the soluble fraction of BL21 cell lysate. Cells were induced at OD_{600} 0.4-0.6 with IPTG for 1hr and lysed using BugBuster. Pellets were re-suspended with 1X Sample Buffer and 10% of boiled samples were loaded as insoluble fraction. 2% and 5% of supernatants were loaded as soluble fraction of lysate. Protein bands were visualized by commassie blue staining. Bands corresponding to GST and GST-LMO4-LID are indicated with arrows. Black lines between samples indicate that samples were run on the same gel but extra lanes were removed. M: Marker.

To increase amounts of soluble GST-LMO4 obtained in the cell lysate, we induced BL21 cells at OD_{600} with IPTG for 2 hrs. Indeed, amount of GST-LMO4-LID increased in the soluble fraction of lysate (Figure 3.35).

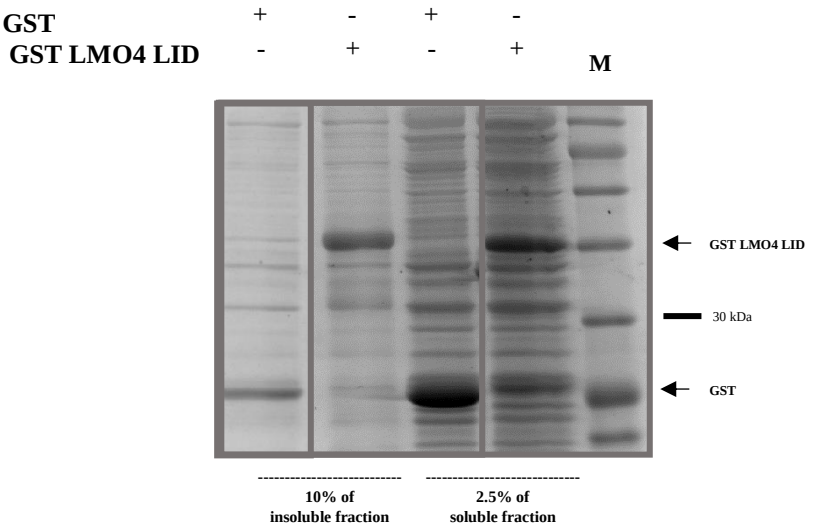

Figure 3.35 : GST-LMO4-LID is in the soluble fraction of BL21 cell lysate. Cells were induced at OD_{600} 0.4-0.6 with IPTG for 2hr and lysed using BugBuster. Pellets were re-suspended with 1X Sample Buffer and 10% of boiled samples were loaded as insoluble fraction. 2% and 5% of supernatants were loaded as soluble fraction of lysate. Bands corresponding to GST and GST-LMO4-LID are indicated with arrows. Protein bands were visualized by commassie blue staining. Black lines between samples indicate that samples were run on the same gel but extra lanes were removed. M: Marker.

HEK293T cells were then transfected individually with plasmids expressing HA-tagged NFIs and HA-tagged LDB1. These lysates were allowed to bind GST-LMO4-LID or GST. In addition, HA-tagged LDB1 was used as negative control. GST-LMO4-LID bound to HA-tagged NFIX, whereas GST failed to bind. The other NFI members could not be pulled down by GST-LMO4-LID. Since LMO4-LID fusion protein has no LDB1 dimerization domain (Figure 3.36), precipitation of LDB1 by LMO4-LID was not observed.

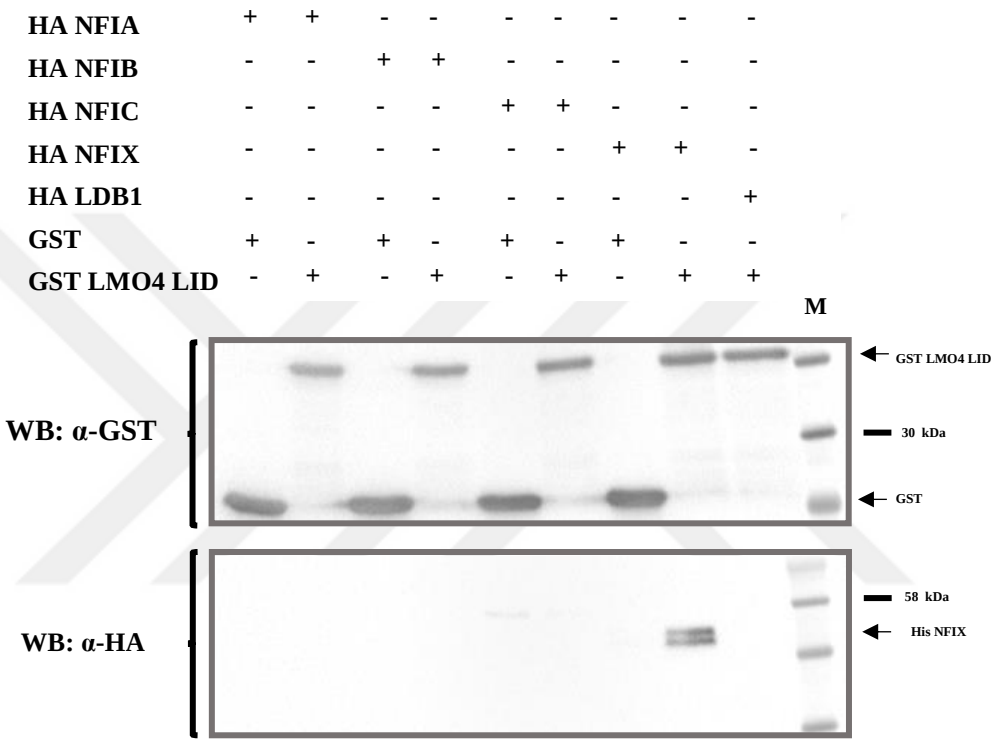

Figure 3.36 : Pull down analysis of LMO4 LID and NFIs. 150 µg NFIA, NFIB, NFIC and NFIX were incubated 2 hrs with 20 µg GST tagged LMO4 LID or 40 µg GST alone, previously immobilized on glutathione sepharose resin. M: Marker.

Together these results indicate that LMO4 preferably interacts with NFIX in HEK293T cells when these two proteins are overexpressed, although we cannot rule out LMO4 interactions with other NFI members.

4. DISCUSSION AND CONCLUSION

In this study, we investigated interactions between NFI and putative binding partners PIN1 and LMO4 using co-immunoprecipitation and GST pull down assays. PIN1 is a peptidyl-prolyl isomerase that is ubiquitously expressed by eukaryotic cells. PIN1 has been previously shown to induce conformational changes of target proteins by catalyzing cis/trans isomerization of pSer-Pro bonds. These conformational changes in PIN1 substrates catalyzed by PIN1 enables PIN1 targets to interact with new partners.

Co-immunoprecipitation assays show that PIN1 interacts with all NFI members when they are co-expressed in HEK293T cells. Additionally, GST pull down assays indicate that bacterially expressed GST-PIN1 could precipitate each NFI. Both co-immunoprecipitation and GST pull down assays found that C-terminal domain of NFIB is not sufficient for NFI binding to PIN1. Consistent with this finding, site-directed mutagenesis of NFIB C-terminal domain indicated that substitutions of putative serine residues to alanine did not abolish, but may have diminished PIN1-NFIB interactions. Taken together, these results suggest that N-terminal DNA binding and dimerization domain of NFI is required for PIN1 interaction, while the other residues located in the C-terminal domain may still contribute to NFI-PIN1 binding. The remaining Ser-Pro PIN binding motifs located in the N-terminal domain could be subjected to site-directed mutagenesis in order to investigate their role in these interactions.

While PIN1 was previously identified as a phosphorylation dependent PPlase that recognizes pS/T-P motif (Shen et al., 1998), phosphorylation independent recognition by PIN1 has also been identified (Pan et al., 2009; Nechama et al., 2013). We found that phosphatase treatment did not affect NFIB and PIN1 binding suggesting that either phosphorylation sites on NFIs are not accesible to the phophatase enzyme due to the association with and steric hindrance by PIN1 or in fact, PIN1-NFIB interactions are not phosphorylation dependent. Alternatively, pre-treatment of lysates with alkaline phosphatase may also have been incomplete as we were unable to include a positive

control that would test for enzyme activity in this experiment, rendering these results as inconclusive.

LMO4 is an adaptor protein that forms multi protein complexes that regulate gene expression. Data obtained from co-immunoprecipitation assays indicate that NFIX and LMO4 bind each other when they are overexpressed in HEK293T cells. Previously, LMO4 was shown to interact with another neurogenic transcription factor, Neurogenin 2, and the interaction between these proteins was found to be strengthened by addition of LDB1 (Asprer et al., 2011). In contrast, if binding surface of LDB1 and the other interaction partner, such as mDEAF1, is the same, LDB1 prevents binding of LMO4 to its partner by competing for LMO4 (Joseph et al., 2014). To address potential regulation of NFI-LMO4 interactions by LDB1, we examined NFI and LMO4 interactions both in the presence or absence of overexpressed LDB1 in co-immunoprecipitation assays. In each experiment, we could show LMO4-LDB1 binding as well as NFIX-LMO4 interactions. However, interactions between NFIX, LMO4 and LDB1 as a multiprotein complex was not detected. These data imply that LDB1 may bind LMO4 and interfere with NFIX interaction. In order to isolate LMO4 in its soluble form, we fused LMO4 with LID domain of LDB1 and used this fusion protein in GST pull-down experiments. LMO4-LID fusion protein still maintains its interaction with NFIX, suggesting that LID by itsef is not sufficient to interfere with NFI-LMO4 interactions. We could not detect any binding between NFIB and LMO4 regardless of the presence or absence of LDB1. Since NFIB or N-terminal domain of NFIB did not interact with LMO4, we surmised that C-terminal domain of NFI may be involved in LMO4 interactions. The differences in NFI family members for LMO4 binding is consistent with this notion. To further investigate this hypothesis, NFIX C-terminal domain can be tested for LMO4 binding in co-immunoprecipitation and GST pull down assays. Moreover, chimeric fusion proteins which comprise N-terminal domain of NFIB and C-terminal domain of NFIX could be created by domain swapping. These chimeras can be further investigated for their ability to bind LMO4.

In this study, we used lysates of HEK293T cells overexpressing NFIs and NFIB prey protein(s) alone or together with bacterial cell lysates that contain prey proteins and assayed for interactions *in vitro*. In both cases, other proteins found in the lysates may participate in interactions of investigated proteins. To test whether NFIs and binding partners directly bind each other, a cell-free translation system, which enables us to

understand direct association between bacterially expressed interaction partners and cell-free translated NFIs, can be used.

In order to show that these interactions are in fact functional, we would like to overexpress or knockdown these proteins and look for effects on NFI's ability to activate target genes and bind regulatory regions etc. PIN1 is highly expressed in the CNS, ventricular zones and neural progenitors during development where NFIs are also present (Nakamura et al., 2012). Knock-out studies show that in the absence of PIN1 neuronal differentiation is significantly inhibited. These defects have been linked with changes in stability of β-catenin in neural progenitor cells. However, PIN1 is likely to regulate other TFs during development as this protein controls many processes and has many substrates. Thus any involvement in NFI function *in vivo* has not been investigated, also the phenotype of PIN1 knockout has yet to be comprehensively studied (Nakamura et al, 2012).

During embryonic development, LMO4 is expressed within the ventricular zone in neuroprogenitor cells (Lee et al., 2005) and within the cortical plate (Sun et al., 2006). Knock-out studies show that LMO4 is an essential factor for cortex development in mice (Huang et al., 2009). In the absence of LMO4, neurogenesis in cortex is impaired and development of functional areas in cortex is inhibited (Asprer et al., 2011; Huang et al., 2009). Studies show that LMO4, together with its interacting partner LDB1, modulates transcription factors by interacting with them, and regulate gene expression mediated by these transcription factors (Joshi et al., 2009; Asprer et al., 2011). Thus, it will be interesting to investigate whether LMO4 plays a role in modulating NFI function *in vivo*.

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APPENDICES

APPENDIX A: Equipment used in this study are listed together with Supplier Companies.

APPENDIX B: Commercial kits used in this study listed together with Cat. No and Supplier Companies.

APPENDIX C: Chemicals used in this study are listed together with Cat. No and Supplier Companies.

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

APPENDIX A

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

Equipment	Supplier Companies
Autoclave	Zealway, GR
Centrifuge	ScanSpeed mini centrifuge, Labogene Beckman Coulter Allegra TM 25R VWR MicroStar 17R Beckman Coulter, A46473 Thermo IEC CL10
Chemidoc	BIORAD, Chemidoc TM MP System
Freezer	Panasonic Ultra Low Temperature Freezer, MDF-U76V
Magnetic Stirrer	Wisd, MSH-20A VWR, VS-C77
Microplate reader	BIORAD, Benchmark Plus
Microscope	Olympus, M021
Nucleic acid spectrophotometer	NANODROP, ThermoFisher Scientific
Orbital shaker Hepa Model	Thermo, 481
pH meter	Inolab wtw
Rotator	Stuart, SB3
Rotator Drive	Stuart, STR4
Shaker	Heidolph, Duomax 1030
Shaker	Sartorius, Certomat SII
Spectrophotometer	Shimadzu, UV-1700
Thermal Cycler	BIORAD T100
ThermoShaker	Biometra, TS1
Vortex	Heidolph, ReaxTop Harmony, LMS Group
Water Bath	ElektroMag, M96KP Memmert, WB110

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

Equipment	Supplier Companies
Agarose Gel Electrophoresis System	BIORAD, Sub-Cell GT Agarose Gel Electrophoresis System
Electronic pipettor	CAPP Controller
Gel Blot Paper	GB003
Hemocytometer	Marienfeld, Neubauer improved
Incubator	Binder, CB150
Microfuge Tubes	Axygen (1.5 ml, 0.6 ml, 0.2 ml) Nest (2 ml, 1.5 ml, 0.6 ml)
Micropipettes	Gilson 10 µl, 20 µl, 100 µl, 200 µl, 1 ml Socorex 10 µl, 200 µl, 1 ml
Microwave	Altus
Power Supply	BIORAD
SDS-PAGE System	BIORAD, Mini-Protean Tetra Cell
Serological pipettes	Sarstedt (25 ml, 10 ml, 5 ml, 2 ml) CAPP (25 ml, 10 ml, 5 ml)
Transfer System	BIORAD, Mini Trans-Blot Electrophoretic Transfer Cell
Tubes	Sarstedt (15 ml, 50 ml) Citotest (15 ml) Isolab (50 ml)
UltraCruz Nitrocellulose Membrane	Santa Cruz, sc3724

APPENDIX B

Table B : Commercial kits used in this study listed together with Cat. No and Supplier Companies.

Commercial Kits	Cat No.	Supplier
20X Lumiglo Reagent and 20X Peroxide	7003	Cell Signaling Tech.
GeneJet Plasmid Maxiprep	K0492	Thermo Scientific
GeneJet Plasmid Miniprep	K0503	Thermo Scientific
In-Fusion HD Cloning Kit	639648	Clontech
NucleoBond Xtra Maxi	740410	Macherey-Nagel
NucleoSpin Gel and PCR Clean-up	740609	Macherey-Nagel
NucleoSpin Plasmid DNA Purification Kit	740588	Macherey-Nagel
Pierce ECL Western Blotting Substrates	32106	Thermo Scientific
Pierce® BCA Protein Assay Kit	23227	Thermo Scientific
QuikChange Multi Site Directed Mutagenesis Kit	200515	Agilent
QuikChange Site Directed Mutagenesis Kit (XL)	200521	Agilent
Western Bright ECL	K-12045	Advansta
X-tremeGene HP Transfection Reagent	06366236001	Roche

APPENDIX C.

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

Chemical	**Cat No**.	**Supplier Companies**
Acrylamide (40%, 29:1)	161-0146	BIO-RAD
Agar	MC006	Labm
Ammonium Persulfate (APS)	BP179-100	Fisher Scientific
Bromophenol Blue	BP115	Fisher Scientific
BugBuster®Extraction Reagent	70584	Merck
D-Glucose Anhyrdous	0188	Amresco
di-sodium hydrogen phosphate	7558-79-4	Merck
Ethanol absolute	4146082	Carlo Erba
Glycerol	800689	MP
Glycine	808822	MP
Nonfat Dry Milk	9999	Cell Signaling
Nonidet®P40	A1694	AppliChem
Potassium dihyrdrogen phosphate	7778-77-0	Merck
SDS	194831	MP
Sodium chloride	7647-14-5	Merck
Sodium chloride	31434	Sigma-Aldrich
Tetramethyl-ethylenediamine	T7024	Sigma
Tris base	BP152-1	Fisher Scientific
Triton X-100®	BP151-100	Fisher Scientific
Trizma® base	T6066	sigma
Tryptone	MC005	Labm
Tween® 20	C58H114O26	Fisher Scientific
Yeast extract	MC001	Labm

APPENDIX C Cont.

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

Chemical	Cat No.	Supplier
Agarose	50004	SeaKem LE
Asetic Acid	6052	J.T.Baker
Coomassie Brilliant Blue	A3480	AppliChem
Dimethyl Sulfoxide	BP231	Fisher Scientific
Ethylenediamine Tetraacetic Acid	BP118	Fisher Scientific
Hydrochloric Acid (37%)	403872	Carlo Erba
Methanol	24229	Sigma Aldrich
Ponceau-S	BP103	Fisher Scientific
Pronasafe Nucleic Acid staining dye	CK130	Conda Lab
Sodium hydroxide pellets	480507	Carlo Erba
0.05% Trypsin-EDTA 1X	25300-054	Gibco
DMEM	319025-CL	Multicell
DMEM	BE12-614F	Lonza
DPBS w/o Ca, Mg	P04-36500	PAN
FBS	10270106	Gibco
L-Glutamine 200 mM 100X	25030-024	Gibco
PBS w/o Ca, Mg	BE17-516F	Lonza
Pen Strep	15140-122	Gibco

APPENDIX D.

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

Buffers and Enzymes	Cat. No.	Supplier Companies
10X 1 Buffer	B7001	New England BioLabs
10X 2.1 Buffer	B7202	New England BioLabs
10X 3.1 Buffer	B7203	New England BioLabs
10X CutSmart Buffer	B7204	New England BioLabs
Alkaline phosphatase, calf intestinal	M0290S	New England BioLabs
BamHI endonuclease	R3136	New England BioLabs
Bgl II endonuclease	ER0081	Thermo Scientific
BsrGI endonuclease	R0575	New England BioLabs
Buffer G	BG5	Thermo Scientific
CloneAmp HiFi PCR Premix	638500	Clontech
KpnI endonuclease	R0142	New England BioLabs
MassRuler DNA Ladder Mix	SM0403	Thermo Scientific
MassRuler Low range DNA Ladder	SM0383	Thermo Scientific
NotI endonuclease	R0189S	New England BioLabs
NotI-HF endonuclease	R3189	New England BioLabs
Protein pre-stained marker	P7712	New England BioLabs
Protein pre-stained marker	P7709	New England BioLabs
Q5 ® High Fidelity DNA Polymerase	M0491	New England BioLabs
XbaI endonuclease	R0145S	New England BioLabs
XhoI endonuclease	R0146V	New England BioLabs
XmaI endonuclease	R0180S	New England BioLabs

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