INVESTIGATING MOLECULAR MECHANISMS OF TRAF7 MUTATIONS IDENTIFIED BY GENOME-WIDE ANALYSIS

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ABSTRACT

INVESTIGATING MOLECULAR MECHANISMS OF TRAF7 MUTATIONS IDENTIFIED BY GENOME-WIDE ANALYSIS

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Meningiomas are defined as the most common primary intracanial neoplasms which originate from meninges. Recent genomic studies identified critical driver mutations in several genes including TRAF7 (TNF Receptor-Associated Factor 7) . Previously, studies performed in Prof. Gunel's laboratory suggested that mutant forms of TRAF7 protein were more stable, possibly due to change in their ubiquitination level. To begin understanding the underlying mechanisms, we looked into interaction partners of TRAF7. TRAF7 is known to interact with TRAF4 (TNF Receptor-Associated Factor 4) and TRAF6 (TNF Receptor-Associated Factor 6). We show that the binding affinity of TRAF7 G536S mutant to TRAF4 was lower compared to other TRAF7 mutants and TRAF7 wild type. However, the interaction between TRAF7 mutants and TRAF6 was not significantly different than that of wild type TRAF7. In addition, we showed lower ubiquitination of K27 and K29 sites on TRAF7 mutants compared to the wild type TRAF7. Interestingly, ubiquitination level on K63 site of mutant TRAF7 wa not significantly altered. Given that TRAF4 and TRAF6 have ubiquitin ligse functions, we think it may be possible that TRAF4 is responsible for K27 and K29 ubiquitination

whereas TRAF6 ubiquitinates K63 of TRAF7. Future studies investigating detailed mechanism of TRAF7 ubiquitination by its interacting partners may help to better understand meningioma and to discover novel therapeutic targets to treat this challenging disease.

Keywords: Meningioma, Molecular Genetics, Ubiquitination

ÖZ

GENOMİK ANALİZLER SONUCU BELİRLENEN TRAF7 MUTANT VERSİYONLARININ MOLEKÜLER MEKANIZMALARININ ARAŞTIRILMASI

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Menenjomlar beyin zarından kaynaklanan ve en sık görülen intrakranyal tümörlerdir. Yakın zamanda yapılan genomik araştırmalar sonucu menenjom patholojisinde önemli bir çok gende yeni mutasyonlar tespit edilmiştir. Bu önemli genlerden biri de TRAF7'dır. Daha önceki çalışmalarımızda TRAF7'nın mutant versiyonların daha dayanıklı olduklarını ve toplam ubikutin seviyelerinin daha az olduklarını saptamıştık. TRAF7 proteinin TRAF4 ve TRAF6 proteinleri ile etkileşim halinde olduğunu biliyoruz. Araştırmalarımız sonucunda G536S mutant versiyonu ile TRAF4 arasındaki etkileşimin doğal fenotip TRAF7 ve TRAF7'nın diğer mutant versiyonlarına nazaran azaldığını tespit ettik. Fakat TRAF7 ve TRAF6 arasındaki fiziksel etkileşimin bu mutasyonlardan etkilenmediğini gözlemledik. MEKK3 varlığında TRAF7 mutasyonlarında K27 ve K29 noktalarında görülen ubikutin seviyeleri azalırken K63 noktasında bu seviye değişmedi. Literaturdeki kaynaklardan TRAF4 ve TRAF6 proteinlerinin E3 ligase aktivitesi olduğunu biliyoruz. TRAF4'un TRAF7'nın K27 ve K29 noktalarındaki ubikutininden sorumlu olduğunu düşündük çünkü TRAF4'a olan

bağlanma eğilimi mutant versiyonda azalırken aynı zamanda K27 ve K29 noktalarındaki ubikutin seviyesi de azalmaktaydı. Hipotezimiz ise TRAF6'in TRAF7'in K63 noktasında ubikutininden sorumlu olduğu yönündeydi. Çünkü mutasyonlar sonucu TRAF6 ve TRAF7 arasındaki etkileşim değişmezken aynı zamanda K63 noktasındaki ubikutin seviyesinde de bir değişiklik gözlemlenmemişti. Araştırmamızda, menenjomlarda tespit ettiğimiz mutasyonlar aracılığıyla TRAF7'ın moleküler mekanizmasını inceledik. Bu çalışma kanser hastaları için yeni terapatik hedefler bulmak açısından önemlidir.

Anahtar Kelimeler: Menenjom, Moleküler Genetik, Ubikutin

To my beloved family and all amazing people who have helped me…

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TABLE OF CONTENTS

LIST OF FIGURES

FIGURES

CHAPTER 1

INTRODUCTION

1.1. Meningiomas

Meningioma is the most common primary intracanial neoplasm which originates from the three-layer meningeal membrane ensheathing the brain and spinal cord in adults. (Figure 1) [1] [2]. Harvey Cushing coined the term Meningioma in 1922. Pathologists later showed that the origin of meningioma is from arachnoid cap cells commonly found in association with arachnoid villi at the dural venous sinuses and veins. [3]

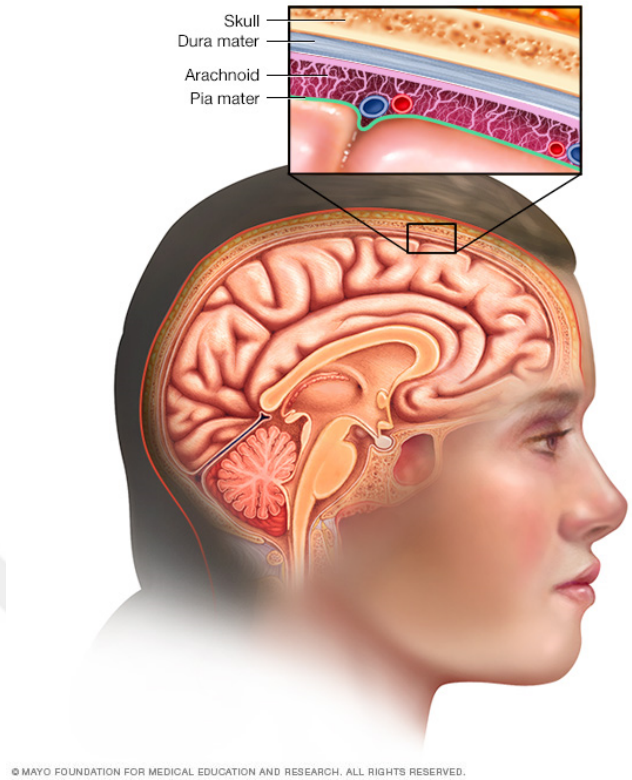


Figure 1 The meninges are the membranous coverings of the brain and spinal cord. There are three layers of meninges: dura mater, arachnoid and pia mater. Courtesy of Mayo Clinic.

The morphological spectra of meningioma shows diversity with both epithelial and mesenchymal features. [4] Meningiomas are generally described according to their location within the brain. The most common are parasagittal, convexity, falcine, olfactory groove, sphenoid ridge, suprasellar and foramen magnum. (Figure 2) [5]

The World Health Organization (WHO) grades meningioma on a scale from I to III according to their histological features such as mitotic activity, cellularity, cellular morphology and growth pattern, necrosis, and brain invasion. For Grade II and III meningioma the rate of recurrence, morbidity, and mortality is higher and they are classified as atypical (5-20%) or malignant (1-3%), respectively. On the other hand

grade I tumors account for approximately 70-80% of meningiomas and they are benign. [2] Grade I tumors have various histological subtypes: meningothelial, fibrous or fibroblastic, transitional (containing both meningothelial and fibroblastic components), psammomatous, microcystic, angiomatous, lymphoplasmacyte-rich, secretory, and metaplastic. Despite the outcome differences resulted by extent of resection, patient age, and position of tumor, it has been observed that the approximate percentage of 10-year overall survival of patients with Grade I meningiomas is 80%-90% [1] Meningiomas have predilection to occur in women at a ratio of 1.7-2.1:1. Since there are hormonal receptors on tumor cells and meningiomas show gender-specific incidence patterns it has been suggested that there is relation between the sex hormones and the pathogenesis of primary brain tumors. However, there is no consistency among the epidemiological studies which explore the association between hormonally mediated risk factors and this gender distribution. [6]

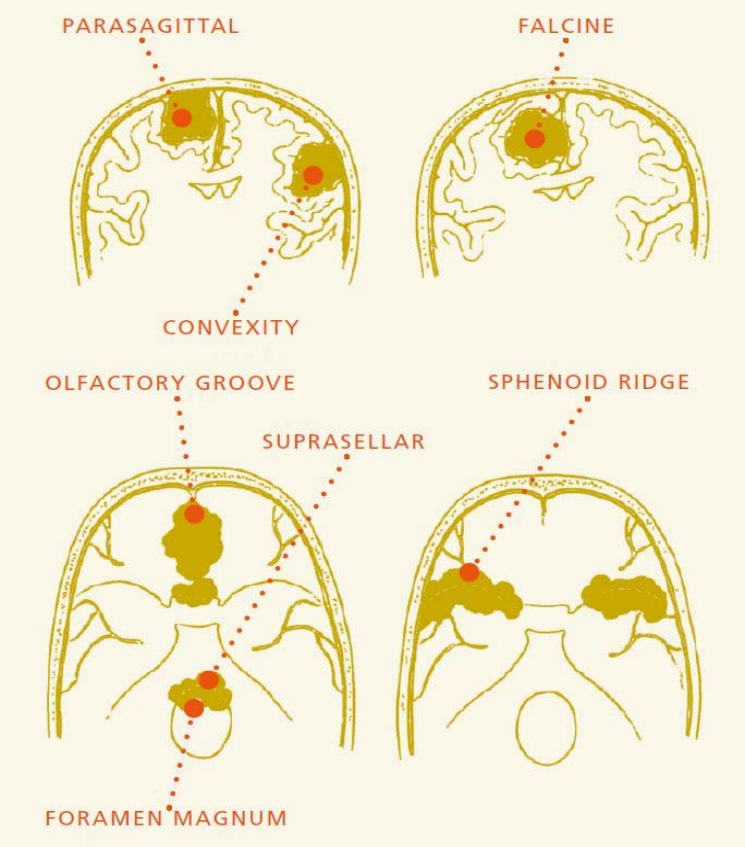


Figure 2 Common locations of meningiomas. (Figure taken from Burger et al, 2002) [5]

Resection is the primary treatment for the patients with meningioma and complete resection is often remedial. Radiotherapy is used for the majority of incompletely resected or recurrent tumors not previously irridiated. Hormonal therapy or chemotherapy are the other possible options in the case the meningioma is unresectable or all other treatments (surgery and radiotherapy) are not successful. It has also been shown that hydroxyurea is efficient to prevent recurrent meningiomas, most likely by resulting in apoptosis in the tumor cells; however, there is a dearth of clinical trials to test the true extent of the success of similar treatments [3].

While most meningiomas are encapsulated benign tumors with limited number of

genetic aberrations, their intracranial location is likely to cause serious and potentially lethal consequences. Unfortunately, there is considerable morbidity related to recurrence which is a challenge for clinicians. [7] [1]

Loss of Neurofibromin 2 (merlin, NF2) is found in 40 to 60% of sporadic meningiomas, but the genetic architecture of the remaining cases is not clear. Recent studies identified several significant gene mutations that are related with these tumors by using unbiased genome- and exome-wide sequencing techniques. An increased mutation burden in TNF receptor-associated factor 7 (TRAF7), Krupple-like factor 4 (KLF4), v-akt murine thymoma viral oncogene homolog 1 (AKT1), and Smoothenedi frizzled family recptor (SMO) has been found in the non-NF2 meningiomas. [8] This information is essential to find novel therapeutic targets and to classify meningioma biologically and more comprehensively [1]

1.2. TRAF Family

The seven TRAF proteins are signal transducing components of the TNF-R superfamily members which transmit a wide range of distinct extracellular signals to the cell and function in the regulation of vital biological processes, including embryonic development and morphogenesis, the innate and acquired immune responses, cell survival and proliferation, tissues homeostasis, and stress responses [9] [10]

TRAF family has seven members named form TRAF1 to TRAF7. All TRAF member from TRAF1 to TRAF6 share a homologous TRAF domain at C-terminal region which functions as a scaffolding region to interact with upstream and downstream effector proteins, as well as to mediate TRAF-TRAF homo/hetero-oligomerization. However, TRAF7 contains a WD40 repeats instead of this characteristic TRAF domain at the C-terminus. Also, all TRAFs except for TRAF1 have a RING domain at N-terminal end. RING domain is associated with the process of ubiquitin-dependent potein degradation

and functions as a E3-like ubiquitin ligase. [11] Ubiquitination is a key regulatory mechanism of TRAFs in signaling.

Some of the TRAF proteins are expressed extremely precisely and dynamically since their spatio-temporal regulation is vital. TRAF3, and TRAF6 has ubiquitious expression while TRAF1 mRNA expression is observed only in spleen lung, and testis tissues. Although TRAF2 and TRAF5 have greatly similar functions and structures, they have different expression patterns. While TRAF2 expression is ubiquitious, TRAF5 is expressed only at significant levels in lung, spleen, thymus, and kidney and at lower levels in liver and brain. [12] TRAF4 shows decidedly dynamic and complex expression pattern during embryogenesis in all species studied (human, mouse, zebrafish and drosophila). Although TRAF4 is expressed in several organs including neural crest cells, salivary gland, thymus, intestine and the epithelium of the trachea according to the developmental stage, in distinct tissues, its overexpression is strongly controlled. This situation may render TRAF4 to have additional tissue-specific function(s). [13] It has wide expression pattern, this may suggest that TRAF4 is pleiotropic and its functions change according to the nature of the cell/organ or even the cell compartment. TRAF7, on the other hand, has two alternative splice forms which are expressed ubiquitiously . [14]

1.2.1. TRAF7

TRAF7 is the most recently discovered 670-amino-acid member of the TRAF family. It has two alternative splice forms which have ubiquitious expression patterns. [14] Endogenous TRAF7 localize to plasma membrane, nucleus, cytosol, nd lysosome. Although it shares an amino-terminal RING finger domain (aa125-160), followed by an adjacent zinc finger domain (aa221-287) with other members of TRAF family, it differs from the other members by seven WD40 repeats at its carboxy terminus instead of the classical TRAF domain. Although the function of TRAF7 is still not clear, it has been shown that there is specific interaction between TRAF7 and MEKK3 and TRAF7

potentiates MEKK3-mediated signaling. [15] Other TRAF family members are able to bind to various signaling molecules, including protein kinases, with their TRAF domain, on the other hand TRAF7 binds to MEKK3 via its WD40 repeats. [16] TRAF7 has been shown to mediate MEKK3 signaling resulting in incresed JNK phosphorylation and apoptosis[14]. The primary signalling pathway shown to be downstream of TRAF7 is the NF-κB pathway with direct interaction reported between both Ikkγ and NF-κB. TRAF7 also sequesters c-Myb to the cytosol via sumoylation and inhibits its trans-activation activity [17]. It has also been shown to affect CHOP medited AP1 activation resulting in apoptosis. TRAF7 and TRAF6 togethe cause lysosomal degradation of anti-apoptotic protein c-FLIP$_L$ via unconventional polyubiquitination. TRAF7 has an essential role in the turnover of c-LIP, subsequently, cell death. [18] It has been shown that TRAF7 function s E3 ligase for the K48-linked ubiquitination of p53. As a result of impairment in the TRAF7-mediated ubiquitination, p53 accumulates. Almost half of human tumors have an elevated level of p53 including breast cancer indicating the essential role of TRAF7 in tumor development and progression. [19]

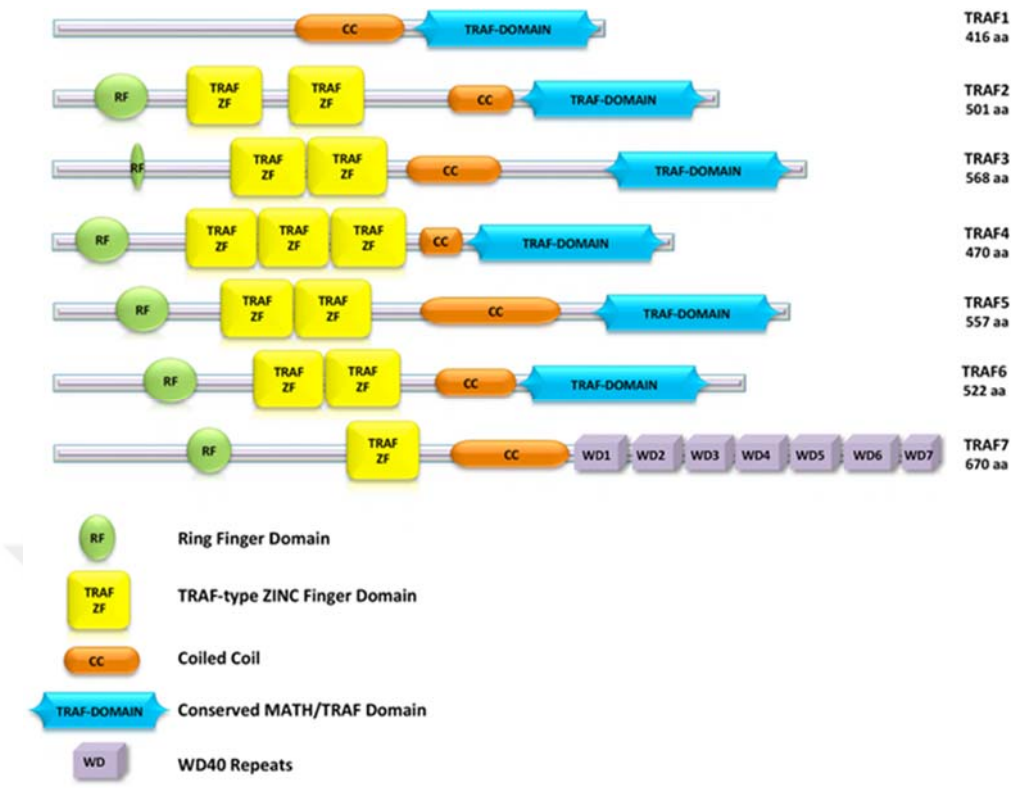


Figure 3 Domain Organization of the seven proteins. (Figure taken from Zottti et al., 2017) [11]

As a result of several recent genome-wide studies of meningiomas researchers have discovered that TRAF7 mutations function as driver mutations and play a critical role in meningioma tumorigenesis. Since TRAF7 WD40 domain function in the interaction of TRAF7 with its partner and subsequently regulate apoptosis these are loss of mutations.

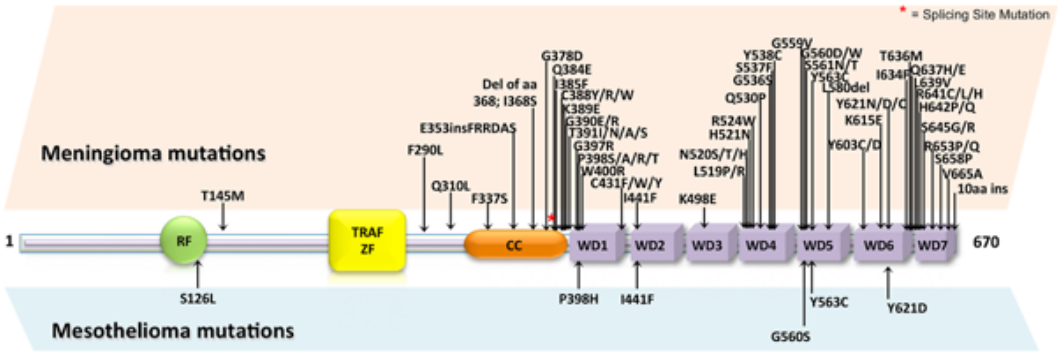


Figure 4 TRAF7 mutations in human cancers (Figure taken from Zottti et al., 2017) [11]

1.2.2. TRAF4

The Tumor Necrosis Factor (TNF) Receptor Associated Factor 4 (TRAF4) gene was identified as a result of differential screening of a cDNA library of breast cancer-derived metastatic lymph nodes and initially called CART1 since it has a cysteine-rich domain associated with RING and TRAF. [20] It is overexpressed not only in breast cancer but in a wide range of human malignancies, including lung cancer, colon adenocarcinomas, melanomas, neurogenic tumors, and lymphomas; therefore, it has been considered as an oncogene. [21]

The fourth member of the TRAF protein family (TRAF4) is distinguished from the other members of the family with several characteristics which concern the primary sequence of the protein, a strong evolutionary conservation, and a tightly regulated physiological expression during development. Except TRAF1, all TRAFs possess an N-terminal RING finger motif; however, TRAF4 (as well as TRAF5 and TRAF6) possess the C3HC3D motif instead of the classical C3HC4 RING motif. [13]

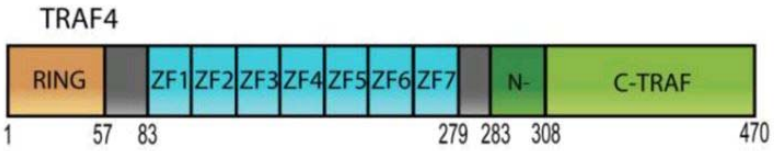


Figure 5 Modular organization of TRAF4. (Figure taken from Rousseau et al., 2011) [20]

Moreover, TRAF4 is the only one which has a nuclear localization signal (NLS). Due to its two additional nuclear localization signal motifs (NLS) in the N-terminal region, TRAF4 exists predominantly in the nucleus although it can also be found in the cytoplasm and cell membrane when overexpressed. Although the other TRAFs contain two CART domains, TRAF4 possesses three CART domains. Also, a second putative NLS exists in the first TRAF4 CART domain. The low capacity of TRAF4 to form heterotypic associations might be caused by the shortness in the coiled-coil domain of the TRAF4, in its N-TRAF domain. This coiled-coil domain of TRAF4 has only three heptad repeats while others have more than ten. Moreover, it has been reported that TRAF4 is not only an adaptor protein but also a regulator protein. Li et al. showed that TRAF4 binds to p47phox, a subunit of NADPH complex, which is critical for NADPH oxidase activation and ROS production [22] Zepp et al. found that there is competition between TRAF6 and TRAF4 to interact with Act1, an E3-ligase NF-κB activator, via the identical TRAF binding sites and regulates IL-17-mediated pathology and signaling pathway. [23] TRAF4 also functins in cell polarity [24]

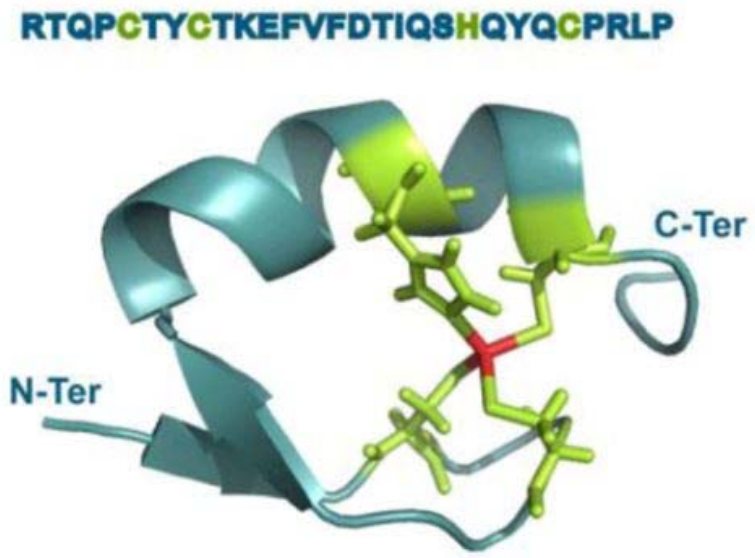


Figure 6 Cartoon diagram of the nuclear magnetic resonance (NMR) solution structure of TRAF4. (Figure taken from Rousseau et al., 2011) [20]

1.2.3. TRAF6

Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) is a unique member of the TRAF family of adaptor proteins since other TRAFs only mediate signaling from the TNFR superfamily, while TRAF6 is involved in both the TNF receptor superfamily and the interleukin-1 receptor (IL-1R)/Toll-like receptor (TLR) superfamily signal transduction pathways. TRAF-C domain of TRAF6 shows the highest divergence. While TRAF-C domain of TRAF2, TRAF3, and TRAF5 recogniz the P-X-Q-X-T motif, it recognizes X-X-P-X-E-X-X-Acidic or Aromatic consensus-binding site. This difference which exists in its TRAF-C domain results in TRAF6 to have distinct physiological functions which regulates a varied array of

processes, including adaptive immunity, innate immunity, bone metabolism and the development of several tissues including lymph nodes, mammary glands, skin and central nervous system. [25, 26] TRAF6 is capable of mediating K63-linked ubiquitination through its E3 ligase activity. It also undergoes autoubiquitination.

1.3. The Ubiquitination

Ubiquitin (Ub), 76-amino acid polypeptide is highly conserved and it shows ubiquitious expression pattern in all eukaryotic cells. Moreover, it has been shown that ubiquitin signaling system exists not only in eukaryotes but also bacteria and archae. [27] Upon binding of this polypeptide covalently, proteins are generally targeted for degradation by the proteosome. However, this is not the only function of ubiquitin. Ubiquitin also involved in regulation of several fundamental cellular processes including autophagy, DNA stability, metabolic pathways, cell cycle, transcription, translation, endocytosis and traffic. [27] Ubiquitin possesses seven lysines (K6, K11, K27, K33, K48, and K63) and the fate of ubiquitinated proteins is determined by the K-type of linkage. This covalent modification called ubiquitination occurs in three enzymatic steps. This process is catalyzed by sequential action of three classes of enzymes; activating enzymes (E1), conjugating enzymes (E2) and protein ligases (E3). [28] Firstly, ubiquitin is activated by adenylation and then formation of a thiol ester bond between the C terminus of ubiquitin and a single cysteine of the E1. [29] After getting delivered to the E2 ubiquitin-conjugating enzyme by E1, ubiquitin is transferred from E2 to lysine resiude in the substrate by E3 ligases. Ubiquitination is a posttranslational modification which can be reversed by special regulatory enzymes called deubiquitinating enzymes (DUBs). Ubiquitin molecules can be detached from the ubiquitin chain iteratively or entire chain can be cut off from the target proteins by DUBs which shows ubiquitin chain specificity [30]. Without antagonistic role of DUBs, several processes in ubiquitin pathway would be disrupted including co-

translational activation of the ubiquitin proprotein, recycle and regeneration of monoubiquitin for succeeding reactions in the cell. [31]

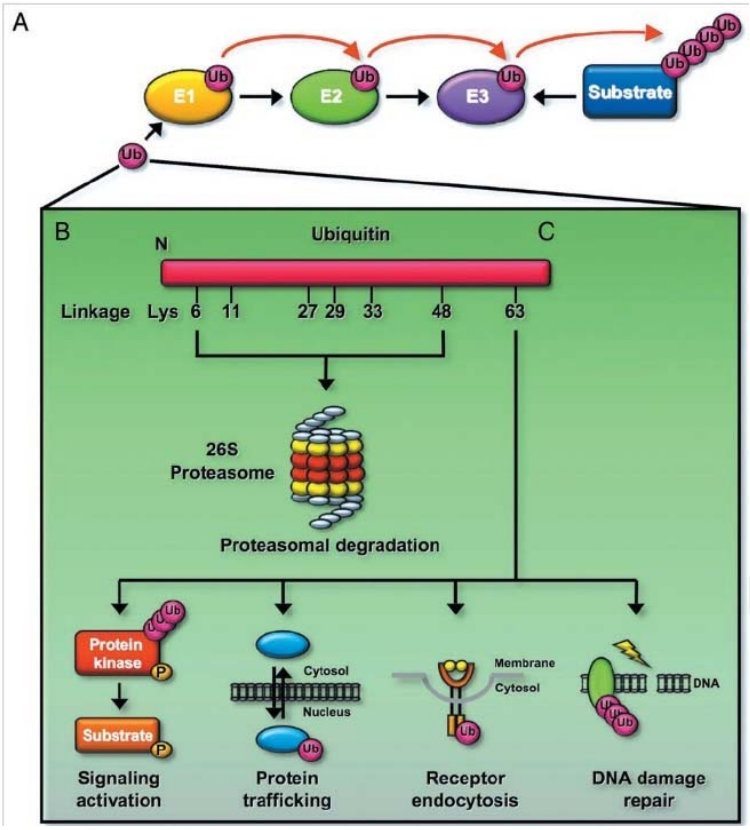


Figure 7 Ubiquitination can mediate protein degradation or activation. (A) Three enzymes function in ubiquitination process. First, ubiquitin is activated and E1 is responsible for this activation. Then ubiquitin is shifted to E2. Proteins recognized by E3 is carried to E2, causing protein ubiquitination. (B) There are seven lysine residues on ubiquitination. The fate of proteins are determined by the type of ubiquitination linkage. For example, protein degradation is mediated by K48-linked ubiquitination, on the other hand, K63-linked ubiquitination regulates signaling activation related with different biological function insted of degradation. Since K63-linked ubiquitination is not recognized by 26S proteasome like K48-linked ubiquitination. (Figure taken from Yang et al, 2010) [32]

Given its role in diverse cellular processes, dysfunction of ubiquitination system may result in cancers, neurodegenerative and immunological disorders. It has been shown that there is direct correlation between the aberrations observed in expression of ubiquitylating, de-ubiquitylating enzymes or Ub-binding proteins and malignancies. [33] As a result of mutations, gene loss or overexpression as well as chromosomal rerrangements Ub modifying eznymes or ubiquitin targets my become resistant to ubiquitylation. In most cases, stabilization of oncoproteins, or destabilization of tumor suppressor gene products through ubiquitination can cause cancers. In addition, deficiency in de-ubiquitinating enzymes can be responsible from the abnormality in growth control and therewith tumorigenesis. This situtation makes investigation of ubiquitination process obligatory to find novel treatments for such diseases. [33]

1.4. NF-κB signaling pathway

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a crucial family of transcription factors which is involved in a wide variety of biological processes, including immune responses, inflammation, cell survival, proliferation and maturation of various cell types. [34, 35] Recent studies increasingly show that NF-κB is a critical player in several steps of cancer initiation and progression. [36] Due to its vital role, there is a need for strict regulation of NF-κB activity. This regulation generally occurs with retention of NF κB in the cytoplasm of unstimulted cell, and its transport to the nucleus when the cells are stimulated. Inhibitory protein of the IκB family sequester NF-κB in the cytoplasm by covering the nuclear

localization signal of NF-κB. IκB kinase (IKK) complex made up by IKKα, IKKβ and NEMO is activated upon stimultion of cells with a broad range of agents, including proinflammtory cytokines such as tumor necrosis factor-α(TNFα) nd

interleukin 1-β (IL-1β), and microbial products such as lipopolysaccharide (LPS). IKK phosphorylates the IκB proteins and results in their degradation via the ubiquitin-proteasome pathway. NF-κB relieved from its inhibitors is then able to enter nucleus and turn on several target genes.

We can broadly divide the NF-κB activation pathway into two pathways as the canonical and noncanonical pathways. This classification depends on whether the pathway include the degradation of IKB or processing of the NF-κB precursors. Although originally thought that these two pathways are distinct, recently several studies have showed that various crosstalk mechanisms connect them. [35]

Ubiquitination/deubiquitination has an essential and complex role in the activation of the NF-κB signalling pathway. For example, phosphorylated USP15 by ATR/ATM cleaves ubiquitin from IκB, thus reducing NF-κB activation. A20 also affect NF-κB regulation via two ways: first taking K63-linked chains away from the RIP1, TRAF2, TRAF6, and NEMO components of this pathway, second facilitating K48-ubiquitination of at least some of these, thereby causing their degradation and suppressing NF-κB activation. [37]

Ubiquitination has a main role in TRAFs signaling. Through K63-linked ubiquitination, TRAFs mediate several non-degradative biological processes including protein interaction, protein trafficking and signal transduction. They are key players in this regulation by functioning as E3 ligase as well as substrate which undergoes ubiquitination. For instance, it has been reported that K63-linked ubiquitination has capability to alter protein function and NF-κB pathway is activated as a result of K63-linked ubiquitination of TRAF6 which is catalyzed by Act1 in IL-17R signaling. [38] Similarly, TRAF6 also ubiquitinates Akt directly at K63 site thereby providing its membrane recruitment and phosphorylation. [39]. On the other hand, K48-linked ubiquitination generally results in degradation of TRAFs through 26S proteasome pathway (Figure 7) [40].

It is increasingly clear that TRAF signaling mechanism need a more detailed study because of the essential role of TRAF proteins as ubiquitin ligases which affects

several signaling pathway including NFKB. [41] They regulate the activation of NFKB and mitogen-activated protein kinases (MAPKs) by TNF receptors (TNFRs), IL-1 receptor (IL-1R) and Toll-like receptors (TLRs). It has been shown that TRAF6 has E3 ligase activity which is responsible of the formation of K-63-based polyubiquitin chains. These nancanonical K-63-based polyubiquitin chains have function in the activation of downstream effectors of TRAF6. Bignell et al investigated the tumors related with the genetic disorder cylindromtosis. When they examined the patients they identified mutations in DUB called as CYLD whose function is to suppress NF-κB activation by deubiquitylating TRAF2, TRAF7 and TRAF6. [42] Yoshida et all also revealed that CYLD interacts with TRAF7 and TRAF6 and that the diminuation in CYLD levels render TRAF6 or TRAF7 more capable to activate NFκB-dependent reporter gene[43] [44]

Although TRAF4 was originaly identified in human breast carcinoma in 1995, its overexpression has since been observed in several human malignancies. However, its mechanism of action has not be explained comprehensively. TRAF4 is associated with numerous signaling pathways. As result of its interaction with nucleotide-binding oligomerization domain 2, innate immune responses are inhibited. It attaches to MEKK4 to medite *c-jun*-NH_2-kinases activation. TRAF4 plays a role in the glucocorticoid-induced TNFR-induced NF-κB activation. TRAF4 is capable to ubiquitinate various substrates via its RING domin which function as E3 ligase. Li et al showed that TRAF4 is the main factor in Akt ubiquitination and activation in lung tumorigenesis. [45]

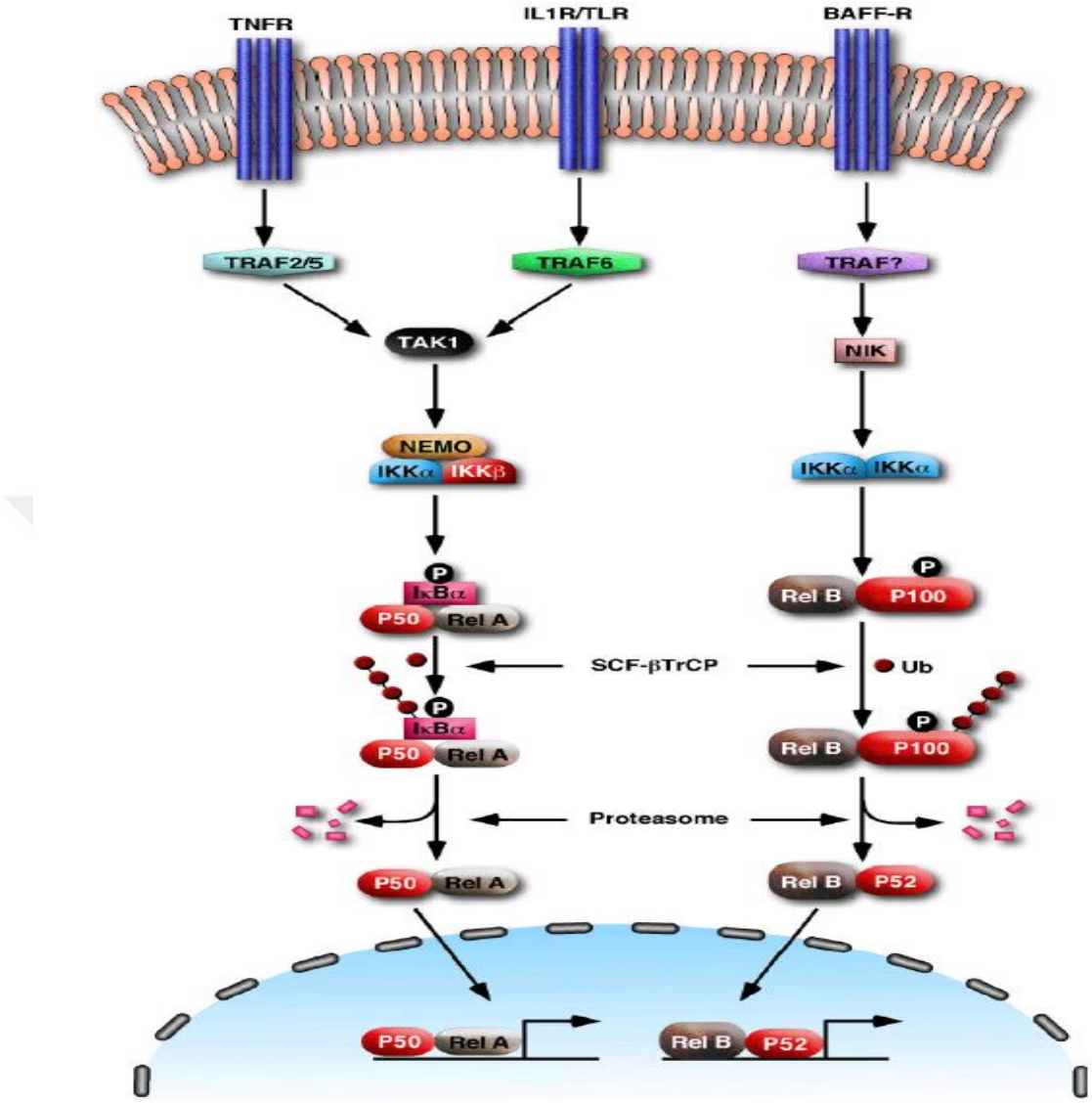


Figure 8 The NF-κB Signaling Pathways. In the canonical pathway (left), upon stimulation of TNF receptors (TNFR), IL-1 receptors (IL-1R) or Toll-like receptors (TLR), TRAF proteins are activated and then TAK1 subsequently. Activated TAK1 phosphorylates and activates IKKβ. As a result, IκB is phosphorylated and ubiquitinated. This ubiquitination cause the degradation of IκB by proteasome. The free p50 and Rel-A, the pieces of NF-κB dimer can translocate to the nucleus in order to mediate the expression of seveal target genes which function in a wide range of biological processes including inflammation, immunity, and cell survival. In the non-canonical pathway, upon stimulation of receptors which belong to the TNFR superfamily, such as the B cell receptor, cause activation of the kinase NIK.

Although the exact mechanism is still not clear, it has been shown that NIK phosphorylates IKKα,which in turn phosphorylates the p100, the NF-κB precursor. Succeeding polyubiquitination of p100 results in its modification by proteasome and it becomes mature subunit p52. Then, p52 can enter into the nucleus with its binding partner to provide expression of several genes. (Figure taken from Chen et al, 2006) [46]

1.5. Specific Aims

Current work by other investigtions in Dr. Gunel's laboratory has characterized three mutant forms of TRAF7: C388R/W/Y, G536S, K615E that occur in meningiomas.[8] The mutations exist in the WD40 domain of TRAF7. Protein ubiquitination studies have shown that the mutant forms are less ubiquitinated and stability assays show that these mutant forms are also more stable (unpublished data). Amongest the previously reported interactors of TRAF7, both MEKK3 and TRAF6 have been reported to play an important role in modulating the effect of TRAF7 in the NFKB signaling pathway.[47] Furthermore, although TRAF7 has autoubiquitination capacity, work in the lab showed that the E3 ligase mutated form of TRAF7, is still ubiquitinated at K27 and K29 (unpublished data). Hence, we tested the hypothesis that either TRAF6 (and potentially TRAF4), both known E3 ubiquitin ligases, could mediate this ubiquitination.

This work addresses the following specific aims:

1. Assess the interaction of TRAF6 and TRAF4 with wt and mutant forms of TRAF7
2. Analyze the ubiquitination of TRAF7 (wt and mutant forms) in response to interaction with TRAF6
3. Elucidate the effect of the interaction of TRAF7 with TRAF4 or TRAF6 on the NFKB signaling pathway

CHAPTER 2

METHODS

2.1. Cell lines, Reagents, and Antibodies

HEK293 (ATCC) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and 1% Penicillin. Stable cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% Penicillin and selection antibiotic G418 (Geneticin). All TRAF7 plasmids used were generated and kindly shared by Dr. Octavian Henegariu. TRAF4 and TRAF6 plasmids were obtained from Addgene. NF-κB-luciferase plasmid was obtained from Agilent Technologies and pRL-Luc was from Promega. All secondary antibodies were from Jackson ImmunoResearch.

2.2. Coimmunoprecipitation and immunoblotting for interaction

HEK293 cells in 60 mm diameter dishes were transfected with constructs. Control plasmid was added in order to keep constant the total amount of plasmid DNA in each transfection. After 5 hours, cells were supplemented with fresh medium. 48 h after transfection, cells were washed with PBS and lysed with a Tris based lysis buffer (pH7.4, 10%glycerol, 1%NP40) containing protease and phosphatase inhibitors and proteins extracted by incubating on a rocker for 20 min at 4°C, the lyates were centrifuged at 12000 rpm at 4°C for 10 min. Supernatants were collected

and exposed to the protein G-Dynabeads beads (Invitrogen) coated with primary antibodies. Proteins were incubated with bead-antibody mixture overnight on the rocker at 4°C. Next day, the beads were washed 3X with Tris buffer then 3X with washing buffer (Invitrogen). Proteins bound to the beads were solubilized in SDS sample buffer in the presence of dithiothreitol. The protein samples were resolved by SDS-polyacrylamide gel electrophoresis, and blotted onto nitrocellulose membranes. For Western analysis, the nitrocellulose membranes were then blocked in 5% non-fat dry milk in 1X TBST (1X TBS + 0.1% Tween) for 1 h at room temperature and incubated with anti-HA (Millipore, 1000-fold diluted), anti-FLAG (Sigma, 1000-fold diluted) anti-MYC (Origene Technologies, 1000-fold diluted) or anti-V5 (Invitrogen, 1:1000) antibodies. The blots were washed three times in TBST and incubated in HRP-conjugated secondary antibody (Jackson Immunoresearch, 1:5000 dilution). After three washes, the proteins were detected by the ECL system (Bio-Rad).

2.3. Ubiquitination Assay

For ubiquitination assays, cells havested from 60-mm dishes were washed once in ice-cold phosphate buffered saline (PBS) before lysis buffer. To detect protein ubiquitination, cells were lysed with 1% SDS in RIPA-modified lysis buffer and proteins were extracted. After incubation for 30 min on the rocker at 4°C, the lysates were centrifuged at 14000 rpm at 4°C for 20 min. After that, the supernatants were switched to new tubes and the cell lysates were diluted with again RIPA modified lysis buffer to reduce SDS to 0.1%. Then, they were exposed to the protein G-Dynabeads Invitrogen) coated with primary antibodies before. The beads were washed with lysis buffer two times then with washing buffer three times. Proteins bound to the beads were solubilized in SDS sample buffer in the presence of dithiothreitol. The protein samples were resolved by SDS-polyacrylamide gel electrophoresis, and then blotted onto nitrocellulose. For Western analysis, the nitrocellulose membranes were blocked

by incubation in 5% non-fat dry milk in 1X TBST solution composed by TBS and 0.1% Tween for 1 h at room temperature and incubated with anti-HA (1000-fold diluted), anti-FLAG (1000-fold diluted) and anti-MYC (1000-fold diluted). The blots were washed three times in TBST and incubated in 1:5000 diluted HRP-conjugated secondary antibody (Jackson Immunoresearch). After three washes, the proteins were detected by the ECL system (Bio-Rad).

2.4. Transfection and NF-κB Luciferase Assay

Before transfection cells were cultured on 96-well plates for 24 h. To evaluate NF-κB activation, HEK293 cells were transfected with each expression plasmid or empty plasmid, reporter plasmid pNF-κB-luc in 96-well plates. All transfections were carried out in triplicate. Where necessary, empty control plasmid was added in order to keep constant the total amount of plasmid DNA in each transfection. To normalize for transfection efficiency, Renilla-luciferase plasmid (pRL-Luc) was added to each transfection. 48 h after, luciferase activity was measured using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions.

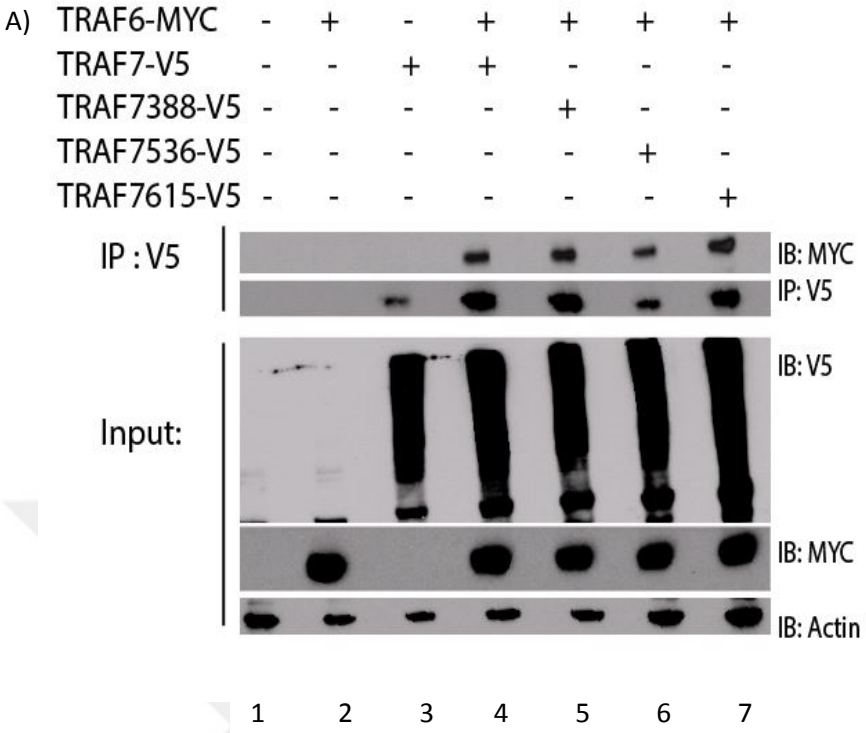
CHAPTER 3

RESULTS

3.1. Physical interaction analysis between TRAF7 and TRAF6

We investigated the interaction of TRAF7 with TRAF6 and the effect of WD domain mutations of TRAF7 on this interaction in HEK293 cells under over-expression conditions. HEK293 cells were co-transfected with V5-tagged TRAF7-WT and mutant versions and MYC-tagged TRAF6 expression vectors or empty vector respectively, then the cell lysates were subjected to immunoprecipitation using anti-V5 antibody. After separating proteins on a polyacrylamide-SDS gel, the Western blot was probed with anti-MYC antibody to detect coprecipitated MYC-TRAF6. Our results shown in Fugre 10 suggested that TRAF7 obviously interacted with TRAF6 and TRAF6 levels in mutant TRAF7 pulled down lysates were higher than that of wild type TRAF7 pulled down samples.

A)


TRAF6-MYC - + - + + + +
TRAF7-V5 - - + + - - -
TRAF7388-V5 - - - - + - -
TRAF7536-V5 - - - - - + -
TRAF7615-V5 - - - - - - +
IP : V5
IB: MYC
IP: V5
Input:
IB: V5
IB: MYC
IB: Actin
1 2 3 4 5 6 7

B)

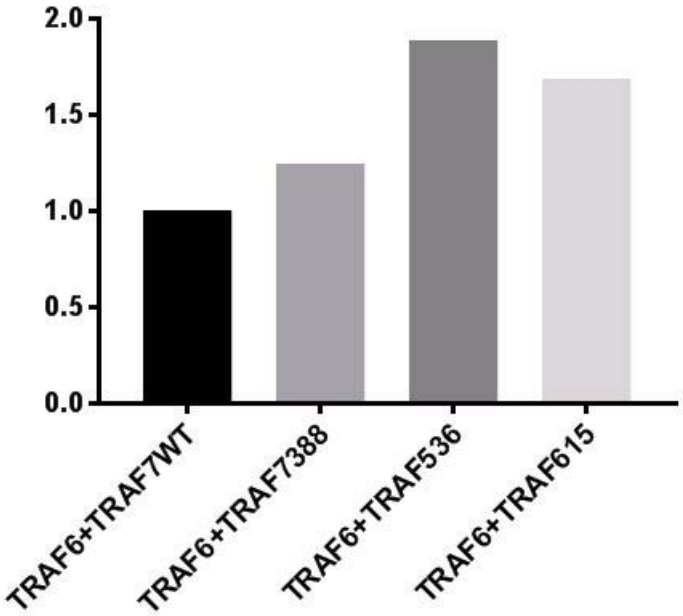


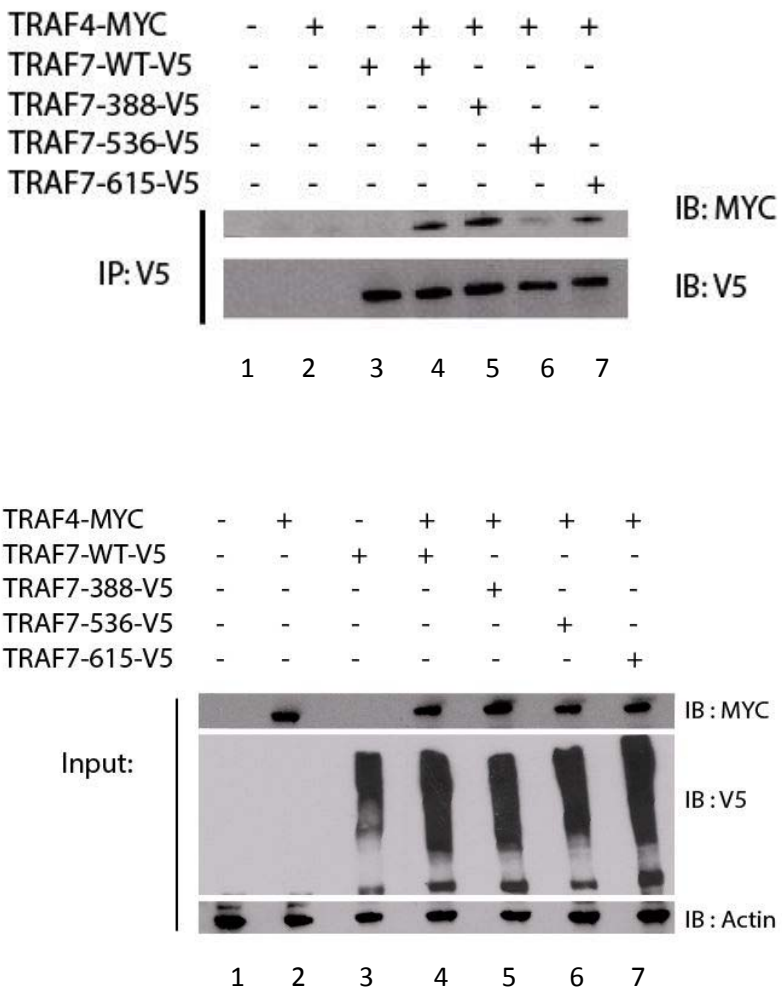
Figure 9 TRAF7 and TRAF6 interaction. A) HEK293 cells were transfected with MYC-tagged TRAF6 along with a vector empty or V5-tagged TRAF7-WT or V5-tagged TRAF7 mutants. 48 h later, cell lysates were immunoprecipitated (IP) with anti-V5 antibody and analyzed by immunoblot probed with anti-MYC. B) Densitometry analysis was done with ImageJ. Experiment was performed three times, a represantative image is shown. TRAF7 nd TRAF6 co-immunoprecipitate. Densitometric analysis shows that mutations in TRAF7 WD40 domain do not affect its interaction with TRAF6.

3.2. Physical interaction analysis between TRAF7 and TRAF4

We investigated the interaction of TRAF7 with TRAF4 in HEK293 cells under over-expression conditions. We also compared the binding affiniy of wild type TRAF7 and TRAF7 mutants (388, 536, 615) toward TRAF4. We co-transfected HEK293 cells with V5-tagged TRAF7-WT and V5-tagged TRAF7 mutant versions and MYC-tagged TRAF4 expression vectors or empty vector respectively, then the cell lysates were

subjected to immunoprecipitation using anti-V5 antibody. After separating proteins on a polyacrylamide-SDS gel, the Western blot was probed with anti-MYC antibody to detect coprecipitated MYC-TRAF4. According to the results shown in Fig.8 TRAF4 levels in TRAF7 mutants pulled down lysates were less than that of wild type TRAF7 pulled down samples. (lane 5,6,7) (Figure 8, A) TRAF4 level was the least in TRAF7 G536S mutant. This effect will be investigated further in the lab. (lane 6) (Figure 8, A)

A)



B)

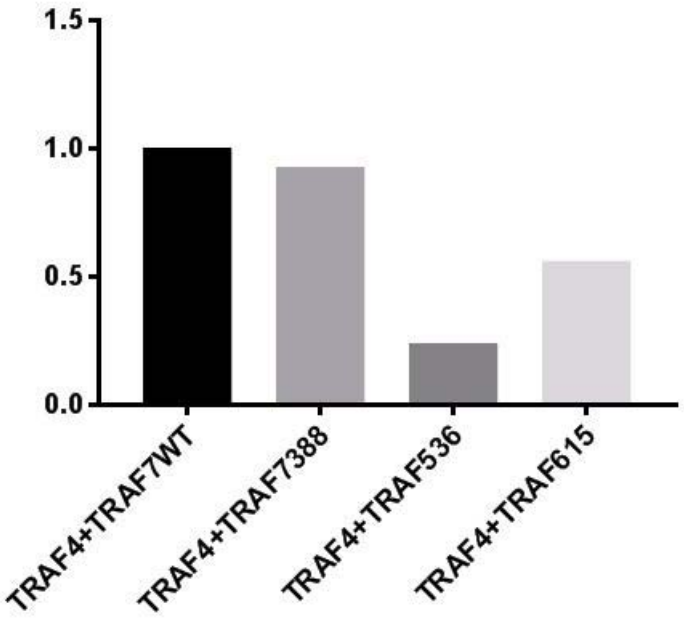


Figure 10 TRAF7 and TRAF4 interaction. A) HEK293 cells were transfected with MYC-tagged TRAF4 along with a vector empty or V5-tagged TRAF7-WT or V5-tagged TRAF7 mutants. 48 h later, cell lysates were immunoprecipitated (IP) with anti-V5 antibody and analyzed by immunoblot probed with anti-MYC. B) Densitometry analysis was done with ImageJ. Experiment was performed three times, a representative image is shown. TRAF7 and TRAF4 co-immunoprecipitate. Densitometric analysis shows that mutations in TRAF7 WD40 domain affect its interaction with TRAF4 G536S mutant of TRAF7 displays most distruption.

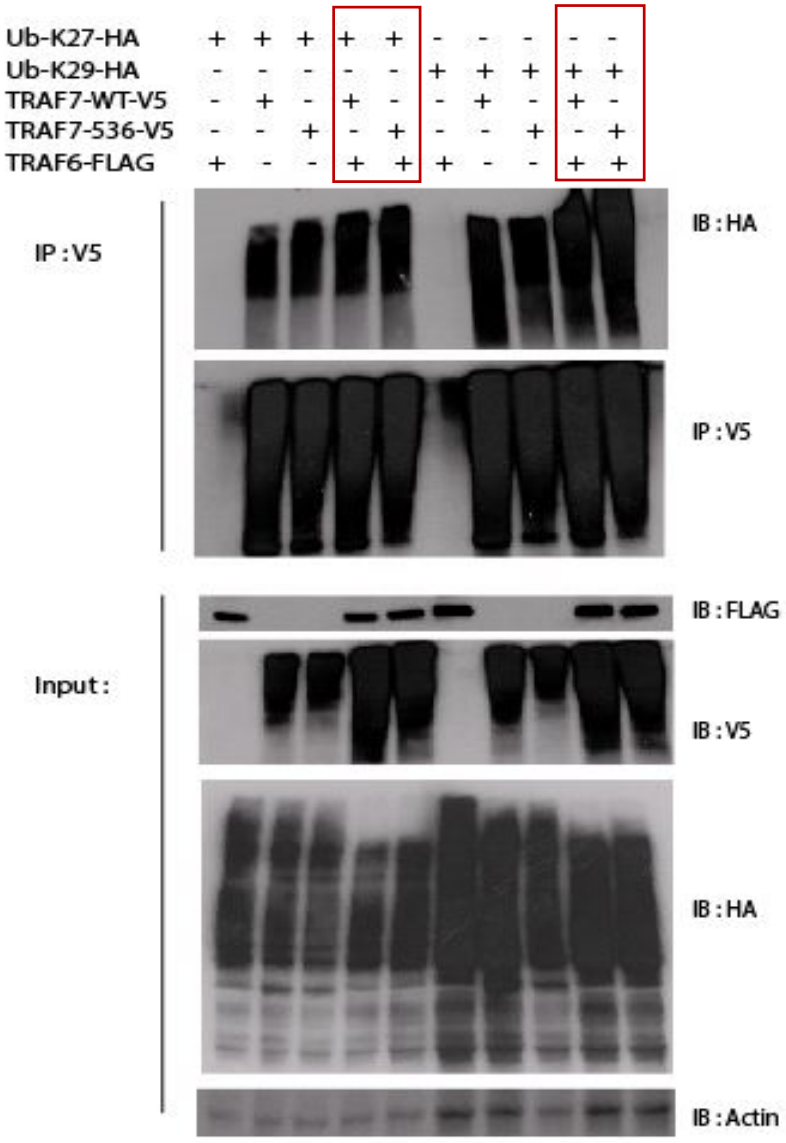
3.3. Ubiquitination assay of TRAF7 at K63 ubiquitination site

Previous data in Dr. Gunels lab has shown that WD40 domain mutants of TRAF7 display an increased half life and reduced ubiquitination in the presence of MEKK3.

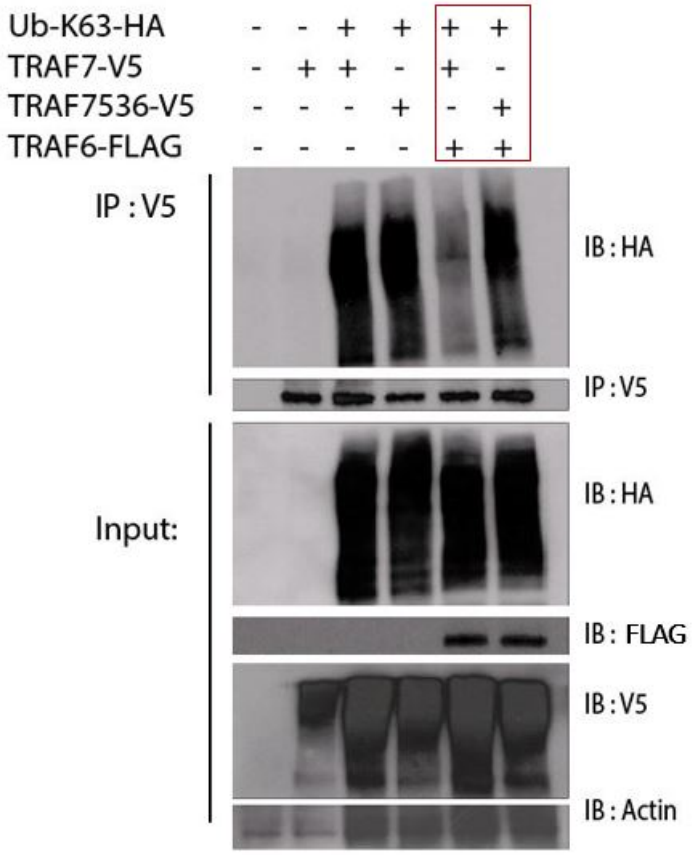
Futhermore, a detailed analysis of this ubiquitinaton showed a reduction in K27 and

K29 ubiquitination – both of which lead to proteosomal degradation. TRAF6 and TRAF4 are both capable of catalyzing K63 ubiquitination – a protein stabilizing form of ubiquitination. As shown our data, we found that the interaction of TRAF7 and TRAF6 is not affected by mutations in TRAF7. Hence, we tested if TRAF7 could be a novel ubiquitination substrate of TRAF6. We first tested the effect of TRAF6 on K27 and K29 ubiquitination of TRAF7 and found that the mutants are not affected in their ubiquitination at both these lysine residues as compared to TRAF7 (Figure 10A). Since mutant TRAF7 does not lose interaction with TRAF6, it is more stable than wildtype TRAF7 and since TRAF6 is known to function as a K63 ubiquitin ligase, we tested if perhaps TRAF6 could be responsible for the K63 ubiquitination of TRAF7. Therefore, we transfected HEK293 cells with exogenous HA-tagged ubiquitin mutants possessing single lysine resiude at K63 for the investigation of the nature of TRAF6-mediated ubiquitination of TRAF7. Interestingly, our results clearly show that TRAF6 does indeed affect the K63 ubiquitination of TRAF7 (Figure 10B) showing that TRAF7 is a novel K63 ubiquitin substrate for TRAF6 and as Figure 10C shows this is independent of MEKK3.

A)


Ub-K27-HA + + + + + - - - - -
Ub-K29-HA - - - - - + + + + +
TRAF7-WT-V5 - + - + - - + - + -
TRAF7-536-V5 - - + - + - - + - +
TRAF6-FLAG + - - + + + - - + +
IP : V5
IB : HA
IP : V5
Input :
IB : FLAG
IB : V5
IB : HA
IB : Actin

B)


Ub-K63-HA - - + + + +
TRAF7-V5 - + + - + -
TRAF7536-V5 - - - + - +
TRAF6-FLAG - - - - + +
IP : V5
IB : HA
IP : V5
Input:
IB : HA
IB : FLAG
IB : V5
IB : Actin

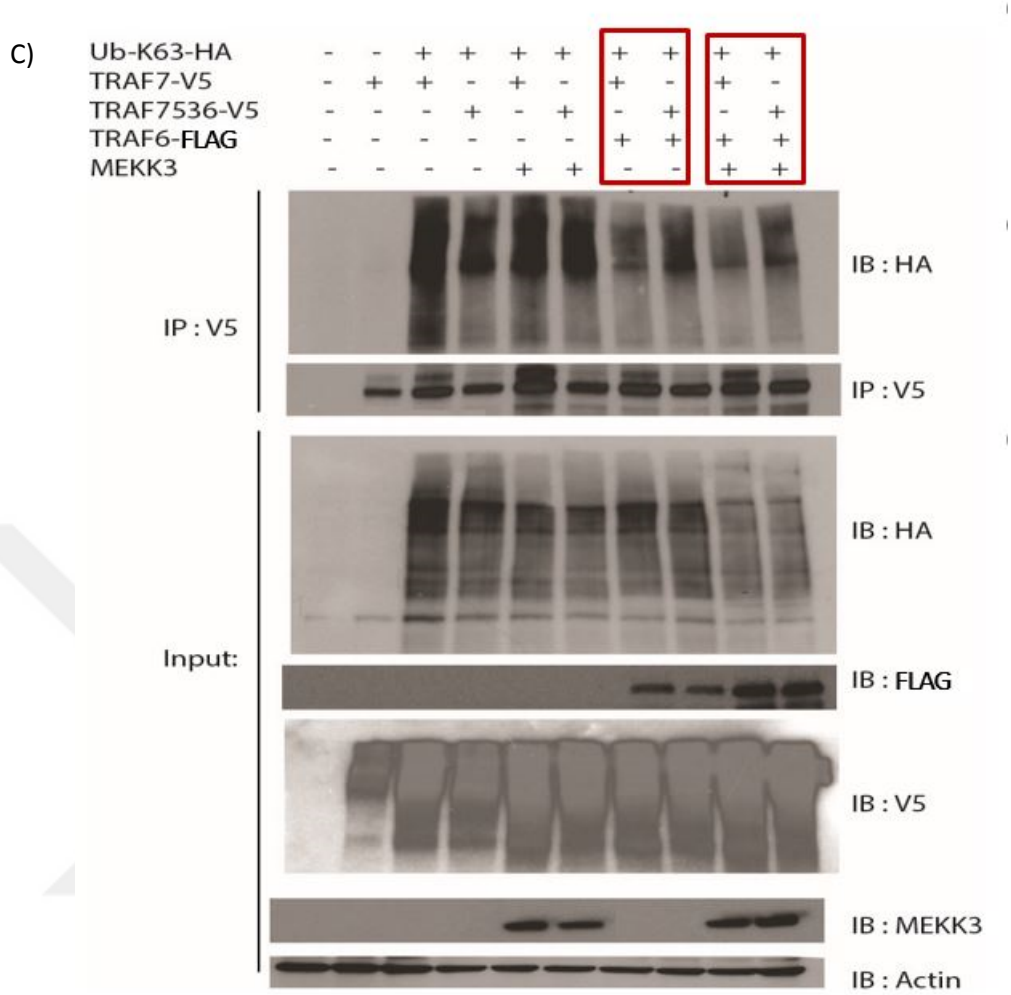


Figure 11 Ubiquitination of TRAF7. A, HEK293 cells were transfected with FLAG-tagged TRAF6, V5-tagged TRAF7 WT and V5-tagged TRAF7 G536S mutant, and HA-tagged ubiquitin mutants possessing single lysine residues (K27, K29). 48 h later cell lysates were immunoprecipitated (IP) with anti-V5 antibody and the ubiquitination state of TRAF7 was analyzed by immunoblot probed with anti-HA. TRAF6 does not ubiquitinate TRAF7 at K27 and K29 sites. B, HEK293 cells were transfected with FLAG-tagged TRAF6, V5-tagged TRAF7 WT and V5-tagged TRAF7 G536S mutant, and HA-tagged ubiquitin mutant K63. 48 h later cell lysates were immunoprecipitated (IP) with anti-V5 antibody and the ubiquitination state of TRAF7 was analyzed by immunoblot probed with anti-HA. TRAF6 suppresses TRAF7 ubiquitination at K63 site; however, TRAF7 G536S mutant is not affected by this suppression C, HEK293 cells were transfected with FLAG-tagged TRAF6, V5-tagged TRAF7 WT and V5-tagged TRAF7 G536S mutant, MEKK3 and HA-tagged ubiquitin mutant K63.

Cell lysates were immunoprecipitated (IP) with anti-V5 antibody and the ubiquitination state of TRAF7 was analyzed by immunoblot probed with anti-HA. The effect of TRAF6 on K63 ubiquitination of TRAF7 is independent of MEKK3. Experiments were performed three times, a representative images are shown.

3.4. Ubiquitination assay of TRAF6

Polyubiquitination has an essential role in TRAF activation, and affects its activity in the NFKB signalling pathway. Given that TRAF6 affects K63 ubiquitination of TRAF7, we then also tested if perhaps the reverse was true and TRAF7 was affecting ubiquitination of TRAF6. TRAF7 has been shown to function as a E3 ligase and primarily catalyzes K29 ubiquitination. We sought to test if perhaps TRAF7 could mediate K63 ubiquitination of TRAF6 and hence we could classify TRAF7 as an E3 ubiquitin ligase capable of mediating K63 ubiquitination, in addition to K29. Therefore, we transfected HEK293 cells with exogenous HA-tagged ubiquitin and detected the ubiqutination state of TRAF6 by immunoblot experiments. Ubiquitin possesses seven lysines (K6,K11,K27,K29,K33,K48, and K63) and the fate of ubiquitinated proteins is determined by the K-type of linkage. Hence, we used a series of ubiquitin mutants possessing single lysine residues for the investigation of the nature of TRAF7-mediated ubiquitination of TRAF6. Interestingly, we found that although TRAF7 does not affect K63 ubiquitination of TRAF6, TRAF7 led to an obvious decrease of TRAF6 K27 and K29 ubiquitination. (Figure 12).

Furthermore, this effect of TRAF7 on TRAF6 ubiquitination is reflected in an increase in the level of endogenous TRAF6 in HEK293 cells stably expressing mutant forms of TRAF7 (Figure 13).

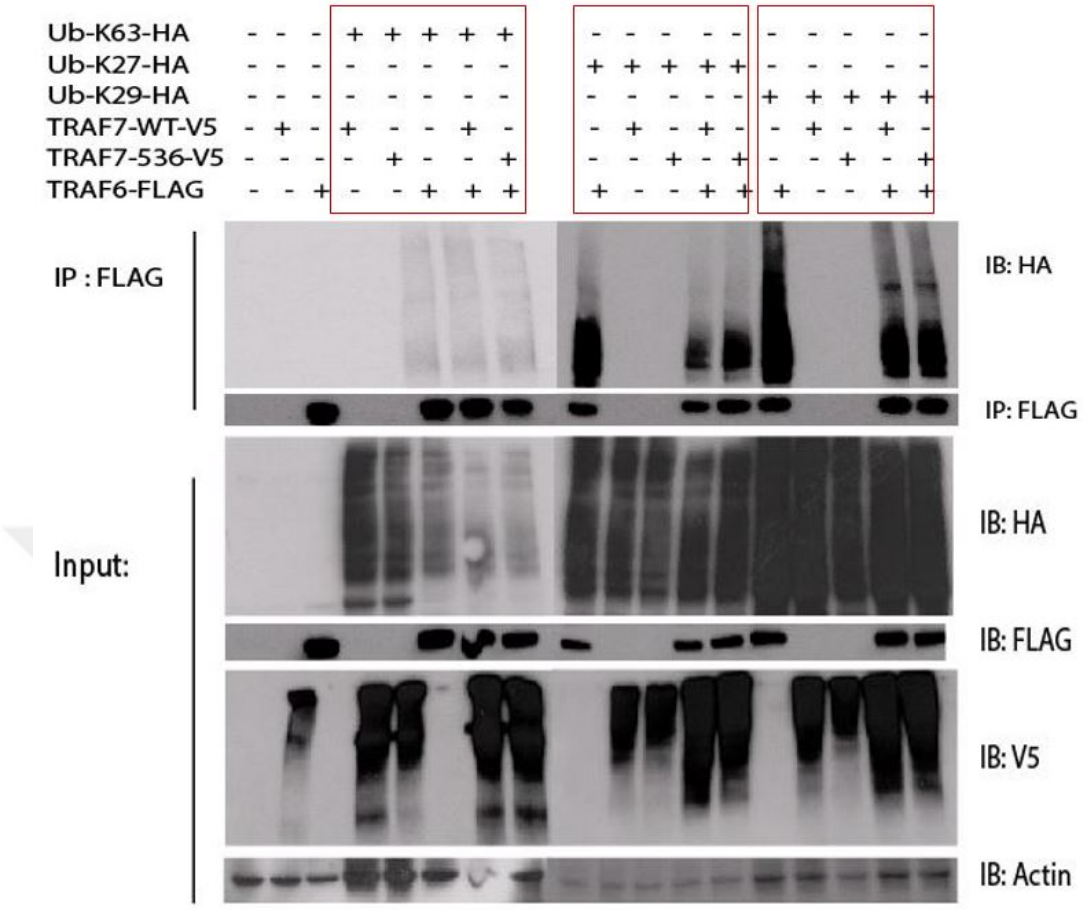


Figure 12 Ubiquitination of TRAF6. HEK293 cells were transfected with FLAG-tagged TRAF6, V5-tagged TRAF7 WT and V5-tagged TRAF7 G536S mutant, and HA-tagged ubiquitin mutants possessing single lysine residues (K27, K29 and K63). Cell lysates were immunoprecipitated (IP) with anti-FLAG antibody and the ubiquitination state of TRAF6 was analyzed by immunoblot probed with anti-HA. TRAF7 suppresses degradative ubiquitination of TRAF6. Experiment was performed three times a representative image is shown.

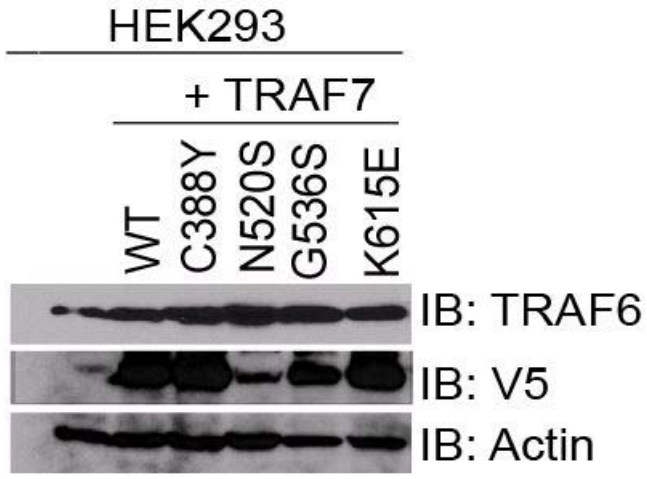


Figure 13 The level of endogenous TRAF6 in HEK293 cells stably expressing mutant forms of TRAF7. Lysates from the indicated HEK293 stable cell lines were immunoblotted with the indicated antibodies. Actin is used as a loading control. Endogenous TRAF6 protein levels are increased in HEK293 cells stably expressing wild type or mutant forms of TRAF7. Experiment was performed three times a representative image is shown.

3.5. TRAF6, TRAF7 and their effect on NF-Kb signaling

Previous reports have shown that TRAF7 and TRAF6 can synergestically activate the NFκB pathway. Mutant TRAF7 displays binding to TRAF6 and furthermore this ability to bind to TRAF6 results in an increase in K63 ubiquitination of TRAF7 and a simultaneous decrease in degradative ubiquitination on TRAF6. Since the overall effect of this would be an increase in both TRAF7 (mutant) and TRAF6 levels, we postulated that this increase in protein levels could contribute to increased NFκB signaling. Hence, we investigated the effect of coexpressing WT or mutant TRAF7 along with TRAF6 and assessed NFκB pathway activation with a NFκB luciferase reporter assay. 48 h after transfection, the cell lysates were subjected for luciferase activities assay. Data are plotted as means ± standard errors for three independent

experiments. We observed that TRAF6 induced activation of NFκB while TRAF7 alone did not induce NFKB activation. Coexpression of TRAF7 with TRAF6 suppressed this induction slightly. However, this suppression was not observed in the TRAF7 mutants. Coexpression of mutants with TRAF6 induce NFKB activation as much as TRAF6 alone.

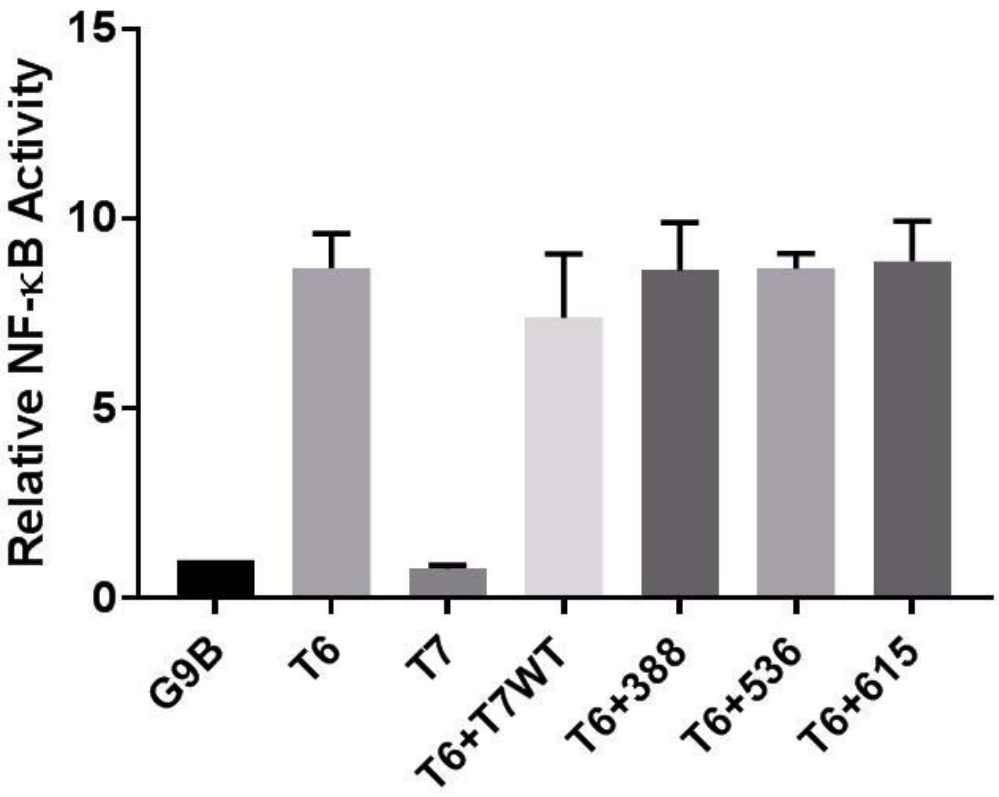


Figure 14 NFkB luciferase reporter assay. HEK293 cells were co-transfected with NF-κB reporters along with empty vector G9B, TRAF6, TRAF7 WT, TRAF7 mutants and TRAF6 with TRAF7 WT or TRAF7 mutants respectively. 48 h after transfection, the cell lysates were subjected for luciferase activities assay. TRAF6 increases the NF-κB activity while TRAF7 was insufficient to increase it. Experiment was repeated three times for the representative image shown, $p < 0.05$.

CHAPTER 4

DISCUSSION

Meningiomas are the most frequent brain tumors which arise from the central nervous system meninges. Although they are nomally slow growing and benign tumors, they have potential for becoming atypical or malignant. Recent research has led to the identification of mutually exclusive molecular subgroups of benign meningioma, including loss of NF2 (occasionally with recurrent mutations in SMARCB1), mutations in the WD40 repeat region of TRAF7 (co-occurring with either PI3K activating mutations or recurrent KLF p.Lys409Gln mutation), activation of Hedgehog signalling (via SMO, SUFU or PRKAR1A), and recurrent p.Gln403Lys and p.Leu438_His439del mutations in the dock domain of POLR2A. It has been shown that these subgroups differ from each other with respect to pathological and clinical features. For instance, TRAF7/KLF4 are responsible for secretory meningiomas, while fibrous meningiomas were primarly affected by NF2 loss. Furthermore, a clinical correlation has been identified between the mutations and anatomical location of meningiomas. Non-NF2 mutant tumors are enriched in the neural crest derived anterior skull base region; on the other hand, samples harbouring NF2 loss arose from the mesoderm-derived posterior regions. Hence, knowing the underlying meningioma mutations makes it possible to predict the intracranial origin of a meningioma and perhaps design targeted therapeutic approaches.

As we previously mentioned, recent research has detected multiple mutations in the WD40 domain of TRAF7. We focused on three of these mutant forms of TRAF7: C388R/W/Y, G536S, K615E that occur in meningiomas. So far, 349 WD40 repeat-containing proteins have been reported to be encoded by human genome. These

beta-propeller proteins function as a scaffold platforms for protein-protein and protein-DNA interactions. Indeed, TRAF7 also uses this WD40 domain in order to establish some molecular interactions. For example, TRAF7 binds to c-Myb and MEKK3 via this WD40 domain. Therefore, the mutations detected in WD40 domain can be important for the interactions of TRAF7 with its partners. In our ubiquitination studies we observed that mutant forms are less ubiquitinated and their stability was higher. (unpublished data). Despite its autoubiquitination capacity, E3 ligase mutated form of TRAF7 is still ubiquitinated at K27 and K29 (unpublished data). Hence, we hypothesized that TRAF6 or TRAF4 was responsible for mediation of this ubiquitination.

When we checked the interaction between TRAF7 and TRAF6, we found that mutant do not lose binding to TRAF6. However, the total ubiquitination was less in the mutant form compared to wild type. Moreover, in the presence of MEKK3, TRAF7 mutant is less ubiquitinated at K27 and K29 but K63 site does not seem to be affected. Since TRAF7 mutant form does not lose its binding affinity to TRAF6 but is still less ubiquitinated at K27 and K29 this suggested that TRAF6 is responsible for the ubiquitination at K63. We hypothesized that TRAF7 can be a novel substrate for TRAF6 for ubiquitination at K63. This might have proved why TRAF7 mutants more stable than TRAF7 wild type. Interestingly, when we performed ubiquitination assays we observed that TRAF7 indeed a substrate of TRAF6 for ubiquitination at K63, and this effect is independent of MEKK3. Furthermore, when we examined the ubiquitination levels on TRAF7 at K27 and K29 sites, we observed that co-expression of TRAF6 did not have any effect. This would explain the increased stabiliy of the mutant forms of TRAF7.

We then also checked whether TRAF7 might be responsible for the K63 ubiquitination of TRAF6. Interestingly, we found that although TRAF7 did not induce K63 ubiquitination it actually suppressed TRAF6 ubiquitination at K27 and K29 sites. Since this might affect TRAF6 stability and consequently its total levels we assessed the levels of TRAF6 in HEK293 cells stably expressing either wildtype or mutant

TRAF7 forms. As expected, this reduction in degradative ubiquitination results in an accumulation of TRAF6 in cells stably expressing mutant forms of TRAF7 as opposed to the WT. It is also possible that binding of TRAF6 to TRAF7 protects it from being degraded by SOCS2, the main E3 ubiquitin ligase involved in K48 ubiquitination and hence degradation of TRAF6. Further studies are needed to address this aspect of the interaction.

Given that TRAF6 was not affecting the K27 and K29 ubiquitination of TRAF7, we also checked the interaction between TRAF7 and TRAF4. We find that TRAF4 is a novel interactor of TRAF7 and interestingly, the G536S mutant displays reduced binding to TRAF4. Since the total ubiquitination of mutant TRAF7 is less than wildtype and this is primarily attributable to reduced K27, K29 ubiquitination (unpublished data), it raises an interesting possibility that TRAF4 is the E3 ubiquitin ligase involved in these degradative ubiquitination events of wildtype TRAF7. Further experiments using E3 ligase mutant forms of TRAF4 will help shed light on this aspect of TRAF4-TRAF7 interaction.

TRAF6 has already been shown to activate NF-κB [48] Moreover, Yoshida et al. reported the synergistic effect of TRAF6 and TRAF7 in which TRAF7 increases TRAF6-induced NF-κB activation. Despite increased levels of both TRAF6 and TRAF7, we do not see this accentuation of TRAF6 mediated NF-κB activation by TRAF7. On the contrary, when we coexpressed TRAF6 with TRAF7, there was suppression in the activation. This may be attributable to differences in the experimental design. The previous study used cell lines which stably express TLR2 as well as cytokine treatment while our study used plain HEK293 cells and did not employ any treatment as we are testing the inherent ability of TRAF7 mutants to overcome the need for TLR2 stimulation.

It is not easy to define the exact role of TRAF7 in meningioma tumorigenesis due to the fact that this protein appears to be involved in such diverse biological processes, sometimes with seemingly opposing functions. However, determining the

functionTRAF7 will be helpful to find novel treatment options for this treatment-refractory ad highly disabling disease

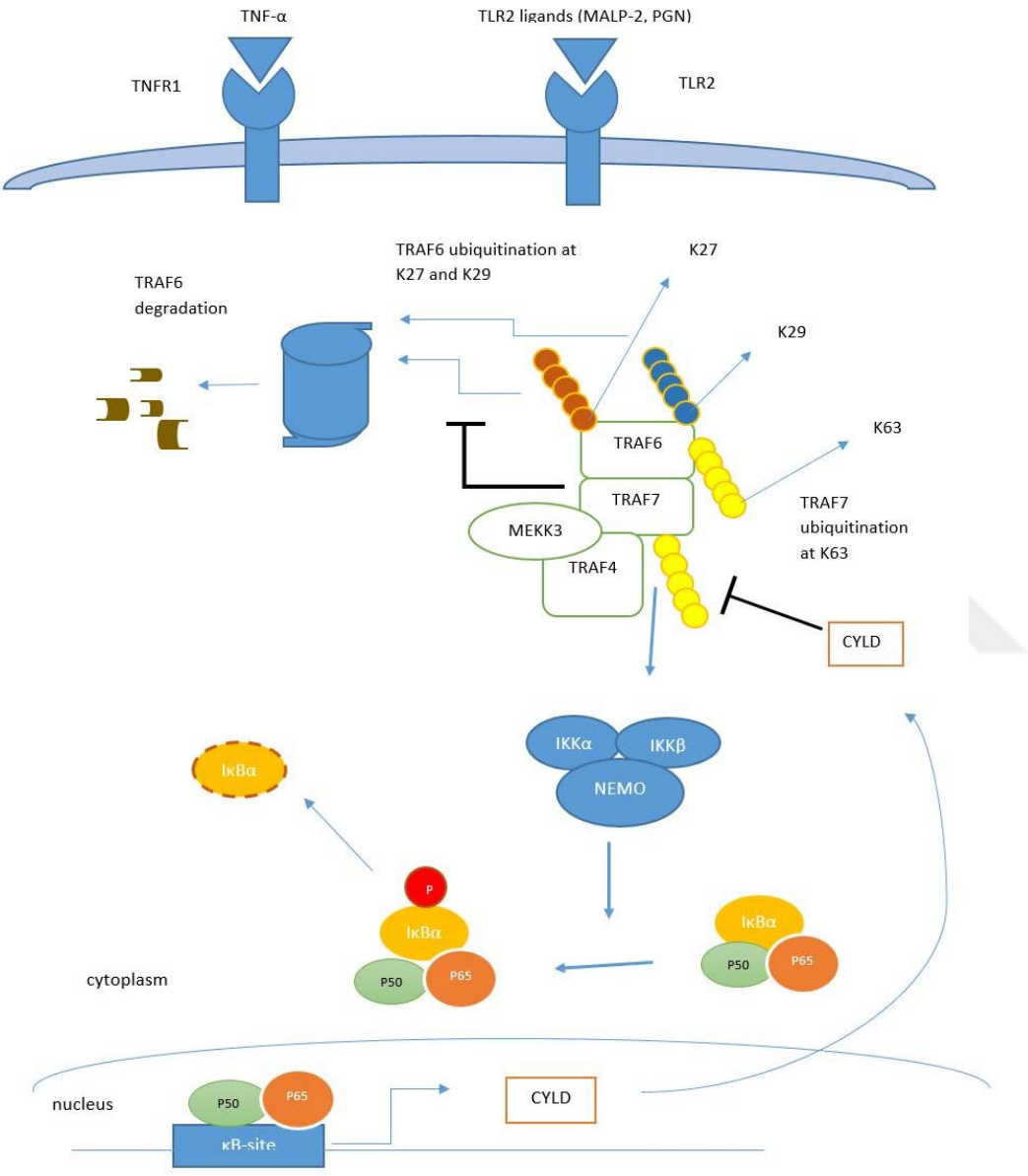


Figure 15 Schematic representation of mechanism between TRAF7, TRAF6, TRAF4 and TRAF4

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