DELINEATING THE INTERACTION BETWEEN TRAF1 AND THE LINEAR-UBIQUITIN CHAIN ASSEMBLY COMPLEX

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Abstract

Tumour necrosis factor receptor (TNFR)-associated factor 1 (TRAF1) is a signaling adaptor that plays opposing roles in NF-κB activation downstream of TNFR and Toll-like receptor (TLR) family members. Furthermore, TRAF1 has been associated with an increased risk of RA, however, the exact mechanisms regarding its role in RA are still unclear. Therefore, isolating its opposing effects on NF-κB activation would provide an excellent model to study the exact role of TRAF1 in-vivo and in disease states such as RA and other inflammatory diseases, where multiple signaling pathways are involved. Since downstream of TLRs, TRAF1 negatively regulates NF-κB by sequestering the Linear-Ubiquitin Chain Assembly Complex (LUBAC), we developed various TRAF1 truncations and mutants and by utilizing co-immunoprecipitation determined the exact protein-to-protein interaction between TRAF1 and LUBAC components. This will help create a TRAF1 mutant that does not associate with LUBAC, while maintaining its role in TNFR and other signaling pathways.

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ACPA	Anti-citrullinated protein antibody
BIR	Baculovirus inhibitor of apoptosis protein repeat
CD	Cluster of differentiation
cIAP	Cellular inhibitor of apoptosis
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
HEK	Human embryonic kidney cells
HLA	Human leukocyte antigen
HOIL1	Heme-oxidized IRP2 ubiquitin ligase
IκB	Inhibitor of κB
IKK	Inhibitor of κB kinase
IL	Interleukin
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon-regulatory factor
JNK	c-Jun N-terminal kinase
K48-Ub	K48-linked polyubiquitination
K63-Ub	K63-linked polyubiquitination
LRR	Leucine-rich repeat
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin chain assemble complex
MAPK	Mitogen-activated protein kinase
Met-1 Ub	Linear polyubiquitination
MyD88	Myeloid differentiation primary response 88
NEMO	NF-κB essential modulator
NF-κB	Nuclear Factor κB
NLR	NOD-like receptor
NOD	Nucleotide binding-oligomerization domain
PAMP	Pathogen-associated molecular pattern
PRR	Pathogen recognition receptor
RA	Rheumatoid arthritis
RBCK1	RANBP2-Type And C3HC4-Type Zinc Finger Containing 1
RF	Rheumatoid factor
RHD	Rel homology domain
RIG-I	Retinoic acid-inducible gene I
RING	Really interesting new gene
RIP1	Receptor-interacting serine/threonine protein
RLR	RIG-I-like receptors
RNF31	RING finger protein 31
SE	Shared epitope
SHARPIN	Shank-associated RH domain interactor
SNP	Single nucleotide polymorphism
TAB	TAK1-binding protein
TAK	Transforming growth factor β-activated kinase
TIM	TRAF-interacting motif

TIR	Toll/IL-1 receptor
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	TNF receptor
TRADD	TNFR type 1-associated death domain protein
TRAF	TNFR associated factor
TRIF	TIR-domain-containing adapter-inducing interferon-β
UBC13	Ubiquitin-conjugating enzyme E2 13
UEV1A	Ubiquitin-conjugating enzyme E2 variant 1A
WT	Wild type

1.0 Literature Overview

1.1 Rheumatoid Arthritis

Rheumatoid Arthritis (RA) is an autoimmune disease characterized by chronic inflammation of the synovium and destruction of cartilage and bone (1). Generally, a progressive disorder that leads to the destruction of the joint and can cause significant pain, severe disability, poor quality of life, systemic complications and increased mortality. It is the most common inflammatory arthritis, affecting about 1% of the population (2). Disease onset occurs between the ages of 30 and 50, with a higher incidence in women, who are 2 to 4 times more likely than men to develop the disease (2,3).

Central to the pathogenesis of RA is chronic inflammation of the joint, however, there are many unanswered questions as to the mechanisms that drive this heightened state of inflammation and how this increased inflammation leads to bone and cartilage destruction. In addition, inflammation is characteristic of the innate arm of the immune system, but the adaptive arm also has a role to play in the pathogenesis of RA. This is evident through the production of autoantibodies in patients with RA, resulting from a loss of self-tolerance (4). These autoantibodies include Rheumatoid Factor (RF), a family of autoantibodies against the Fc portion of immunoglobins and anti-citrullinated protein antibody (ACPA), a family of autoantibodies against citrullinated proteins. RFs are detected in 60-80% of RA patients and ACPAs in 70-90% of RA patients (5,6). However, the presence of these autoantibodies is not always indicative of RA, as they may be present in healthy individuals and individuals suffering from other autoimmune diseases (1,7). Furthermore, mechanistically how these autoantibodies lead to an increased inflammatory state remains unclear. Thus, the etiology of this disease likely involves a complex interplay between many different intracellular signaling pathways and regulatory factors of the immune system.

Furthermore, the heterogenous nature of this disease suggests the interaction between environmental and genetic factors. Indeed, twin studies indicate genetic risk factors, with concordance rates of 15 to 30% among monozygotic twins and 5% amongst dizygotic twins (8). In addition, it has been well-established through genome-wide analyses of a RA-associated gene, the human leukocyte antigen (HLA)-DRB1 locus, which accounts for about 30% of the heritable risk in RA (9). HLA-DRB1 alleles that encode for a five amino acid sequence, known as the shared epitope (SE), in the third hypervariable region of DRβ chains are associated with an increased risk for RA (10). Although this strong relationship exists between the SE and RA, mechanisms through which this SE induces RA are still unknown. Furthermore, the SE only accounts for 30% of the possible 50-60% genetic risk factor for RA, suggesting additional genes involved in the susceptibility to the disease. Therefore, genes regulating diverse intracellular signaling pathways need to be examined. One possible target is nuclear factor-κB (NF-κB) which regulates a plethora of genes involved in both the innate and adaptive immune response (11).

1.2 Nuclear factor-κB (NF-κB)

Nuclear factor-κB (NF-κB) refers to a family of inducible transcription factors responsible for regulating the distinct signaling pathways of the innate and adaptive immune response (12). This family is comprised of 5 members: NF-κB1 (also referred to as p50), NF-κB2 (also referred to as p52), RelA (also referred to as p65), RelB and c-Rel (11). Members of this family share the Rel Homology domain (RHD), which allows them to form hetero- or homo-dimers with each other and bind to a particular DNA sequence, known as the κB enhancer element (13). Grouped into 1 class are members p50 and p52, as they arise from precursor proteins, p105 and p100, through cleavage of their ankyrin repeats, respectively. Grouped into

another class are members, Rel A, Rel B and c-Rel which exist as mature, functioning proteins (14).

NF-κB regulates a plethora of genes involved in the immune response, such as cytokines, chemokines, immunoreceptors and cell adhesion molecules. Thus, its activity is inhibited under resting conditions through binding by a set of inhibitor proteins known as the Inhibitor of κB (IκB) family which keep NF-κB sequestered in the cytoplasm, preventing it from entering the nucleus (13). These members all contain a set of ankyrin repeats (AnkRs) that bind to NF-κB similar to those found in the precursor proteins p105 and p100, which function as IκB-like proteins in these precursors (14). Activation of NF-κB requires removal of this inhibition which can occur through either pathway: canonical and noncanonical (also referred to as alternative), which is dependent on the stimulus or receptor being activated. For our purposes, only the canonical pathway will be discussed as it is the primary pathway through which NF-κB is activated.

The canonical pathway mainly influences p50/RelA and/or p50/c-Rel heterodimers and involves IκBα, the predominant IκB member regulating the canonical pathway (15). This pathway is centralized on IκBα degradation through its phosphorylation by a multi-subunit complex called IκB kinase (IKK). IKK is composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit called NF-κB essential modulator (NEMO) or IKKγ. Upon activation, IKK phosphorylates IκBα at two N-terminal serines which leads to its K48-linked polyubiquitination (K48-Ub), subsequent degradation and results in nuclear translocation by NF-κB (11). In addition to K48-Ub, K63-linked and linear polyubiquitination are important in providing a platform for IKK to be phosphorylated. The mechanism through which IKK is activated is dependent on the stimulus or receptor being activated and such receptors that induce

canonical NF-κB activation include cytokine receptors, pattern recognition receptors (PRRs), TNF receptor (TNFR) superfamily members and antigen receptors (15,16). The pathway is very well understood in a family of PRRs, known as Toll-like receptors (TLRs) and the TNFR superfamily member TNFR1.

1.3 Linear Ubiquitin Chain Assembly Complex (LUBAC)

Ubiquitination is an important post-translational modification involved in regulating a wide array of cellular processes, such as protein degradation, DNA repair, protein trafficking and intracellular signaling (17). Central to this process is the ability of ubiquitin to form a chain of ubiquitin molecules, known as polyubiquitin chains. Ubiquitin is a 76 amino acid protein consisting of 7 lysine residues, each of which can be linked through to form polyubiquitin chains. In addition, ubiquitin molecules can be attached via the N-terminal amino group of methionine, known as Met-1 linked or linear ubiquitin chains, hence, a total of 8 different types of homotypic polyubiquitin chains exist (18). Modification of proteins with ubiquitin requires the sequential transfer of ubiquitin by 3 enzymes: E1, an ubiquitin activating enzyme, E2, and ubiquitin conjugating enzyme and E3, an ubiquitin ligase (19). The most prevalent type of linkage in cells is K48-Ub and its role is to target proteins for degradation by the proteasome. The second most common type, K63-Ub, is involved in signaling and DNA repair (20). Linear ubiquitination, however, is a still relatively novel type of ubiquitination and unlike other types it is only mediated by LUBAC.

LUBAC, the linear ubiquitin chain assembly complex, is an E3 ligase consisting of 3 subunits: HOIP (a.k.a. RNF31) and HOIL1 (a.k.a. RBCK1), the catalytic subunits, and SHARPIN, the non-catalytic subunit (21). Both HOIP and HOIL1 possess catalytic activity, but it is the 2 RING finger domains of HOIP that are essential for linear ubiquitination by LUBAC (21). LUBAC is crucial to canonical NF-κB activation as it synthesizes linear chains to NEMO,

specifically onto Lysine residues in the CC2-LZ domain of NEMO (20). Necessary for linear ubiquitination of NEMO is binding of the LUBAC via the NZF domains of HOIP and HOIL-1L (21,22).

1.4 Toll-like Receptors (TLRs)

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) comprised of 10 members in humans (TLR1-TLR10) which detect conserved structures of diverse microbes, known as pathogen-associated molecular patterns (PAMPs) and endogenous molecules released from damaged or dead cells known as damage-associated molecular patterns (DAMPs) (23). They are expressed in both immune cells and non-immune cells such as fibroblasts and epithelial cells (24). The TLR is comprised of an ectodomain with leucine-rich repeats (LRRs) which detect PAMPs or DAMPs, a transmembrane domain and a cytoplasmic Toll/Interleukin (IL)-1 receptor (TIR) domain which transmits the signal to downstream effectors (25). TLRs are either located extracellularly on the cell surface, TLR1,2,4-6,10, or intracellularly in endosomes, TLR3,7,8,9 (26). As mentioned above, NF-κB activation differs between receptors based on IKK activation. In TLRs, NF-κB activation is divided into MyD88-dependent signaling, which occurs downstream of all TLRs except TLR3, and TRIF-dependent signaling, which occurs downstream of TLR3 and endocytosed TLR4.

Upon stimulation, TLRs form hetero- or homo-dimers. In the MyD88-dependent pathway, MyD88 recruits IRAK4 which simultaneously recruits and activates IRAK1, through phosphorylation (27,28). IRAK1 undergoes autophosphorylation which facilitates TRAF6 recruitment and subsequent TRAF6 oligomerization leading to its activation as an E3 ubiquitin ligase. Associating with the ubiquitin-conjugating enzyme UBC13 and UEV1A, TRAF6 induces K63-linked polyubiquitination of itself and other substrates such as TAK1, TAB1, TAB2 and NEMO, as well as synthesizing free, unanchored K63-polyubiquitin chains (29). The TAK1

complex consists of TAK1 and TAB proteins 1,2, and 3, the TAB proteins interact with unanchored K63-polyubiquitin chains to activate TAK1. TAK1 co-localizes with IKK through the polyubiquitin chains on NEMO and phosphorylates IKKβ leading to its, and, ultimately, NF-κB activation, as mentioned above, as well as other transcription factors including IRFs and AP-1 family members (Figure 1) (26). In TRIF-dependent signaling, TRIF directly recruits TRAF6 and RIP1which induces the TAK1/IKK axis (30).

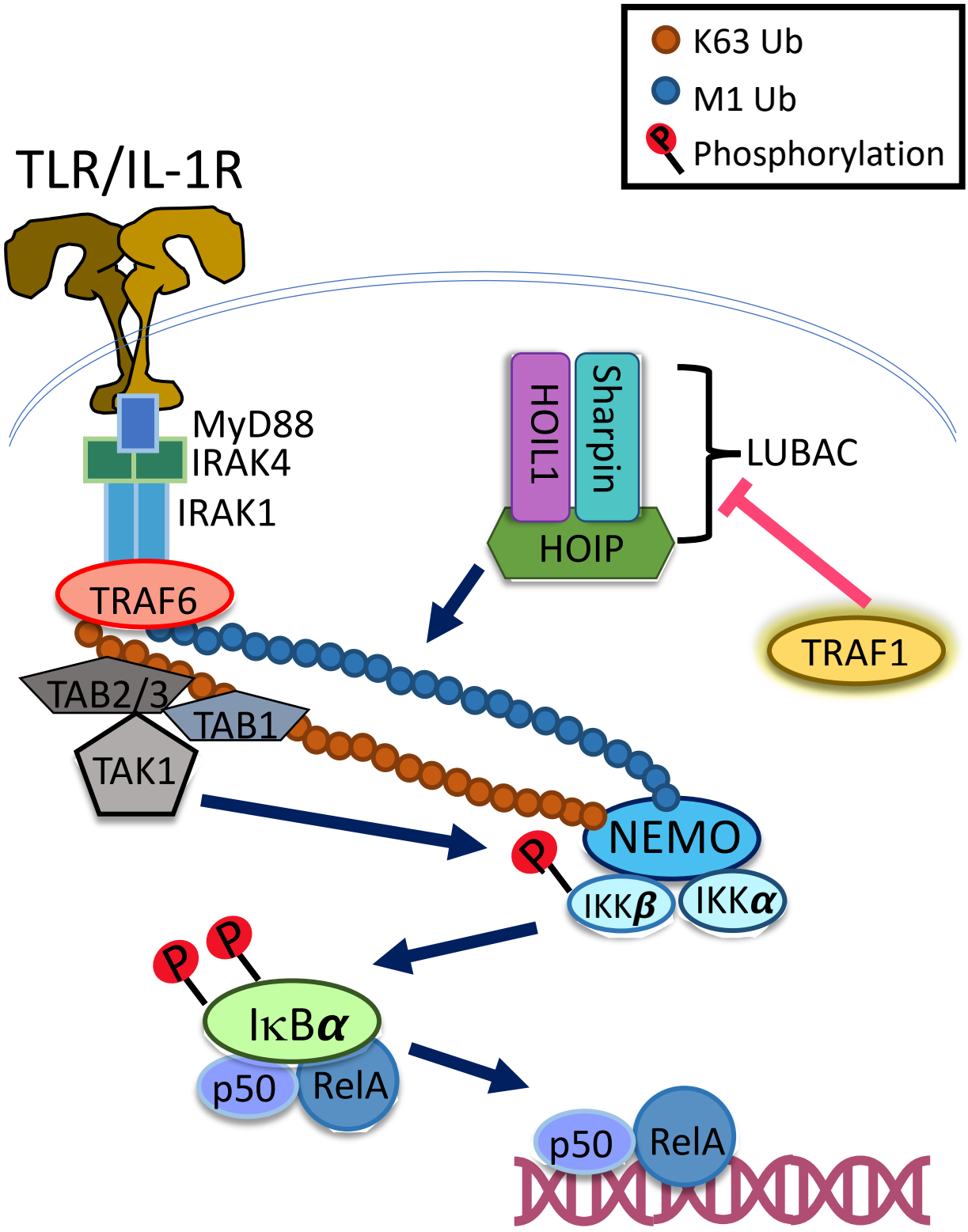


Figure 1. An overview of NF-κB activation downstream of TLR/IL-1R. Image adapted from (31).

1.5 Tumour Necrosis Factor Receptors (TNFRs)

The Tumour Necrosis Factor Receptor (TNFR) superfamily are a class of cytokine receptors that all share an extracellular cysteine-rich domain, which binds to each receptors respective ligand (32). This superfamily of receptors can be categorized into two main groups: death receptors, named after the intracellular region they all possess, the death domain, which helps induce cell death; and TRAF-interacting motif (TIM)-containing receptors which do not contain a death domain but a TIM domain which binds TRAF proteins (33). These receptors do not have kinase activity, hence, they require adaptor proteins to transduce the signal to downstream effectors. These adaptor proteins are specific to the type of receptor; signaling by death receptors occurs through adaptor proteins that contain a death domain, such as TRADD or FADD and by TIM-containing receptors primarily through TRAFs (34).

Although TNFR1 was initially identified as a death receptor, the default signal upon TNF binding is to help induce cellular proliferation and survival (35). Upon binding of TNF to TNFR1 both RIP1 and TRADD, death-domain containing proteins, are recruited to the receptor (36). TNFR1-bound TRADD recruits TRAF2 which in turn recruits cIAP1/2, E3 ubiquitin ligases that catalyze the K63-Ub of RIP1 (36). TRAF1 likely aids in this recruitment of cIAP by forming a heterotrimer with TRAF2, TRAF1:$(TRAF2)_2$, as it has been shown to bind to cIAP2 with a higher affinity than TRAF2 alone (37). TRAF1 and TRAF2 directly interact with the BIR domains of cIAP1 and cIAP2 (38). As a result of K63-Ub of RIP1, the TAK1 complex is recruited, through binding of K63-ubquitin to the TAB2 subunit. LUBAC is also recruited by the K63-Ub of RIP1 and catalyzes the linear-ubiquitination (M1-Ub) of RIP1 and NEMO, which facilitates the recruitment of the IKK complex leading to the phosphorylation of IKKβ by TAK1, resulting in its activation (Figure 2) (39). Linear-ubiquitination catalyzed by the LUBAC is crucial to NF-κB signaling, as NF-κB signaling is suppressed in HOIL-1, member of the

LUBAC, knockout mice (40). After examining both TLR and TNFR signaling, it is apparent TRAF proteins are important in activation of NF-κB downstream of these receptors.

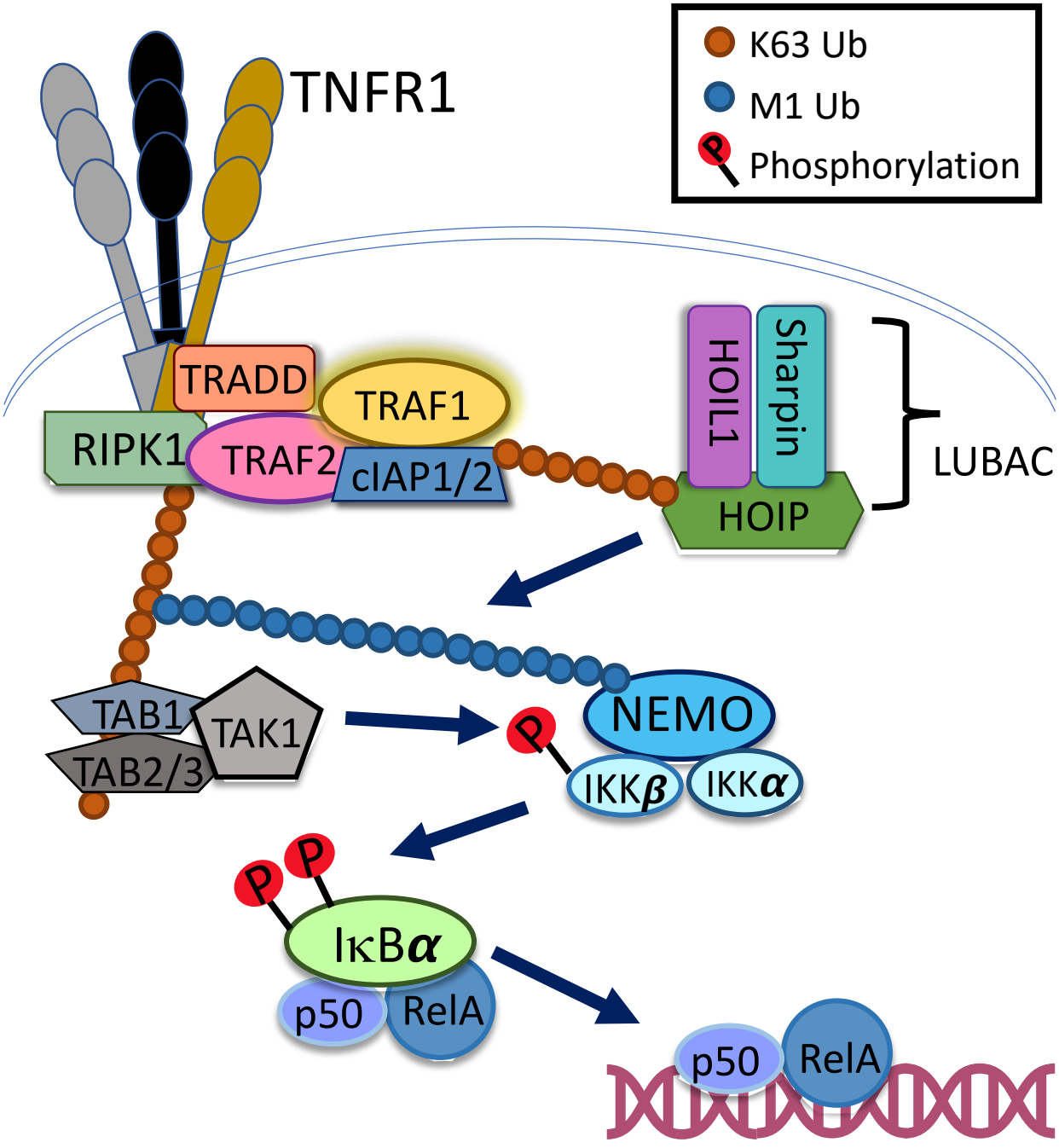


Figure 2. An overview of NF-κB activation downstream of TNFR1. Image adapted from (31).

1.6 TNFR-associated factors (TRAFs)

The tumor necrosis factor receptor (TNFR)-associated factor (TRAF) family of proteins were originally discovered as mediators of TNFR2 signaling, but are now known to be involved in a variety of immune receptor signaling pathways such as the aforementioned toll-like receptors (TLRs), nucleotide binding-oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) and cytokine receptors (41,42). As adaptor proteins, they form receptor-associated signaling complexes, which mediate the interaction between upstream receptors and downstream effector molecules (43). Certain TRAF members have also been known to act as E3 ubiquitin ligases (44,45). As adaptor proteins and E3 ubiquitin ligases, TRAFs ultimately lead to the activation of transcription factors such as NF-κB, mitogen-activated protein kinases (MAPKs; e.g. ERK-1 and ERK-2, JNK and p38) and interferon-regulatory factors (IRF; e.g. IRF3 and IRF7) (46,47).

The TRAF family is comprised of 6 members (TRAF1-6), which all contain a C-terminal TRAF domain (48). The TRAF domain can be divided into a N-terminal coiled-coil region (TRAF-N) and a highly conserved C-terminal Beta-sandwich domain (MATH Domain) (Figure 3) (49). It is the TRAF domain that mediates oligomerization and interaction with upstream receptors and downstream effectors (41). Despite the similarity of the TRAF domain, each TRAF possesses their own unique function through their interaction with distinct signaling receptors and downstream effectors. In addition to their role in immune signaling, they are also involved in embryonic development, tissue homeostasis, stress response and bone metabolism (41).

In comparison to all the other TRAFs, TRAF1 is unique as it lacks a RING finger motif and contains only 1 Zinc finger, thus, resembling a dominant negative form of TRAF2 (44,50). TRAF1 expression is induced by NF-κB signaling and is limited to activated myeloid and

lymphoid cells (51). Despite lacking a RING finger domain, TRAF1 is an important regulatory molecule in NF-κB and MAPK activation, however, TRAF1's effect on NF-κB activation is peculiar, as results from a variety of studies have indicated TRAF1 as both a positive and negative regulator of NF-κB activation (52). These contrary conclusions have led to much confusion in the literature about TRAF1's role in NF-κB activation.

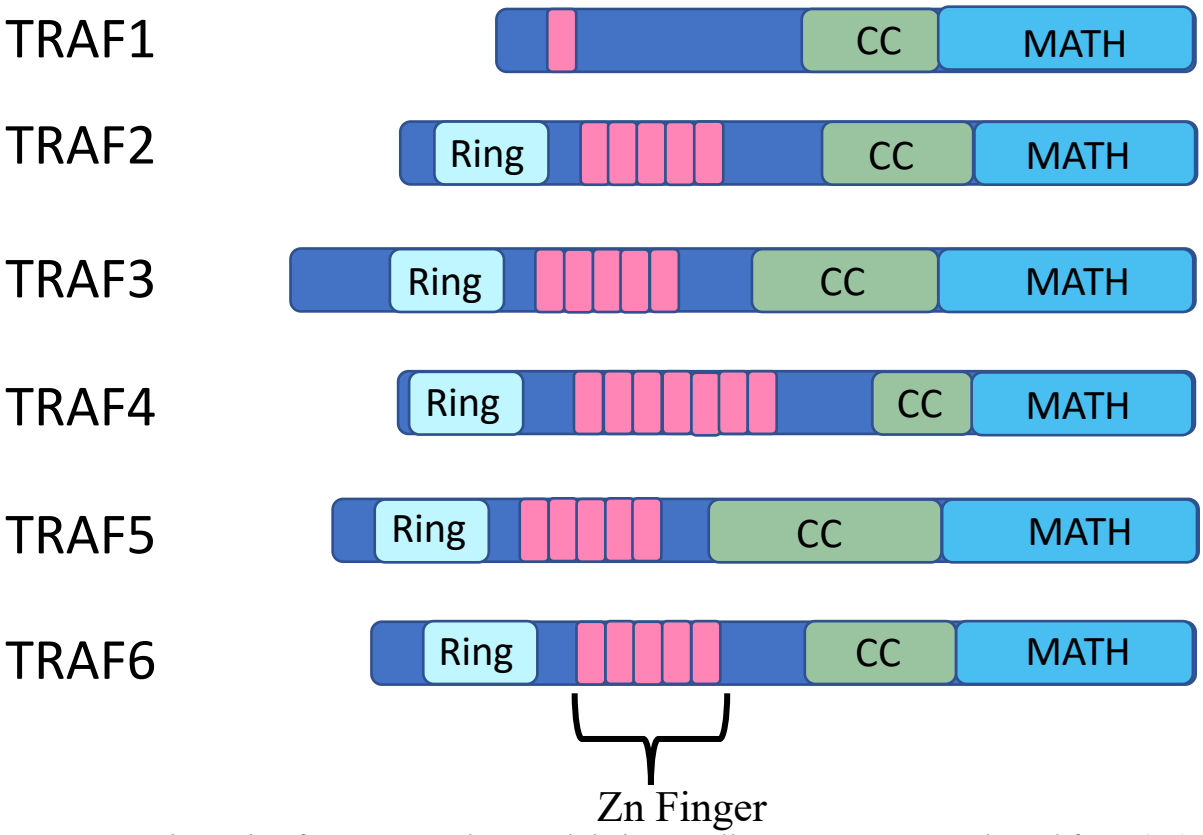


Figure 3. A schematic of TRAF members and their overall structure. Image adapted from (53).

1.7 TRAF1 Signaling downstream of Tumour Necrosis Factor Receptors

Initial studies on TRAF1's role in TNFR signaling suggested it negatively regulated NF-κB activation downstream of TNFR family members, as Carpentier et al. found overexpression of TRAF1 in HEK293T cells prevented NF-κB activation in response to TNF and IL-1 (52). Furthermore, in TRAF1-deficient mice, T cells showed an increased proliferation to anti-CD3 stimulation when compared to WT T cells and it was the TRAF1-/- T cells but not TRAF1+/+ T

cells that responded to TNF by activation of the NF-κB pathway (54). Hence, these studies suggested negative regulation of NF-κB activation downstream of TNFR family members by TRAF1. However, overexpression of TRAF1 in a T-cell-antigen receptor transgenic model resulted in a decrease in antigen-induced apoptosis of CD8+ T cells likely through the induction of NF-κB (55). In addition, it was shown that TRAF1, TRAF2, cIAP1 and cIAP2 were all needed to prevent TNF-alpha-induced apoptosis in the HT1080 fibrosarcoma cell line (HT1080I) (56). This is likely through the formation of a heterotrimer between TRAF1 and TRAF2, as the TRAF1:(TRAF2)2 heterotrimer has been shown to interact with cIAP2 with a higher affinity than TRAF2 alone in TNF signaling (37).

The opposing roles of TRAF1 in NF-κB activation may be dependent on the cell-type and the specific TNFR family member being activated. As TRAF1-deficient dendritic cells show increased apoptosis and decreased NF-κB activation after CD40 stimulation (57). Moreover, using the mouse B-cell-derived cell line, CH12.LX, Xie et al. showed in CH12.LX cells deficient in TRAF1, TRAF1 and TRAF2 cooperate to induce C40 stimulation (58). In addition to forming a heterotrimer with TRAF2 which helps with cIAP recruitment, TRAF1 may be preventing TRAF2 degradation by cIAPs, which have E3 ligase activity for adding K48-Ub (59). As Wicovsky et al. showed in Jurkat/HeLa cells TRAF1 prevented TNFR2-induced degradation of TRAF2 and this prevention allowed for TNFR1-induced activation of NF-κB (60).
In contrast, a caspase-generated cleavage product of TRAF1 has been shown to interfere with TRAF2-mediated survival signaling (61,62). As it was found by overexpressing TRAF1 in HeLa cells lead to inhibition of TRAF2-induced NF-κB activation downstream of CD40 signaling but not TNFR1 (63). Therefore, the negative regulation of NF-κB activation implied in the aforementioned studies may be the result of this cleavage product of TRAF1.

4-1BB, a TNFR family member, is involved in sustaining the survival of activated and memory CD8 T cells through induction of JNK, p38 and NF-κB (64–67). In T cells, TRAF2 is essential for 4-1BB signaling as it is recruited upon 4-1BB ligation (68). Multiples studies suggest TRAF1 positively regulates 4-1BB signaling in T cells (43,61). Indeed, TRAF1 -/- CD8 T cells have aberrant 4-1BB induced NF-κB activation (50). In the same study McPherson et al. found TRAF1 negatively regulates the alternative NF-κB pathway. As discussed above, TRAF1-/- T cells show increased proliferation to anti-CD3 stimulation when compared to WT T cells. It is likely this increased proliferation is a result of a decreased restraint on the alternative pathway. Hence, a large portion of the literature suggests TRAF1, in normal physiological conditions, positively regulates canonical NF-κB activation in T cells by assisting TRAF2, how much that is through forming a heterotrimer with TRAF2 to enhance cIAP2 recruitment or preventing TRAF2 degradation still remains to be answered.

1.8 TRAF1 Signaling downstream of Toll-Like Receptors

Since first being discovered as an adaptor to TNFR2 signaling, TRAF1's role downstream a variety of other TNFR family members has been thoroughly examined, as mentioned above, however, only recently has its role in TLR signaling been elucidated. Recently, Abdul-Sater et al. showed increased NF-κB activation after stimulation with the TLR4 ligand, LPS, in TRAF1 knocked down THP-1 cells, shTRAF1 THP-1 cells (69). Thus, suggesting TRAF1 negatively regulates TLR-dependent activation of NF-κB in THP-1 cells. Mechanistically, it was shown that TRAF1 interacts with LUBAC, reducing linear ubiquitination of NEMO, as evident by the significant increase in M1-Ub, but not K63- or K48 -Ub of NEMO in shTRAF1 THP-1 cells when compared to control. However, TNF-induced NF-κB activation was not increased in shTRAF1 THP-1 cells. This is likely due to TRAF1's role in positively

regulating TNFR signaling through forming a heterotrimer with TRAF2 which recruits cIAP1/2 at the same time leading to K63-Ub of NEMO (37). Indeed, shTRAF1 THP-1 cells upon TNFR1 activation showed an increase in M1-Ub but decrease in K63-Ub of NEMO. Hence, these opposing roles resulted in no change in NF-κB activation. In addition, it was found the interaction between TRAF1 and LUBAC components is mediated by the MATH domain of TRAF1 which binds to all 3 components of LUBAC through their NZF domains, hence, preventing LUBAC from binding to NEMO and catalyzing linear ubiquitin chains.

1.9 TRAF1 and Rheumatoid Arthritis

As mentioned earlier, genetics contributes 50-60% of the risk for RA and although the HLA-gene contributes to 30% of the heritable risk, genes contributing to the remainder of the genetic component are still unknown (61). NF-κB drives transcription of many different genes involved in inflammation, therefore, dysregulation of NF-κB has the potential to lead to chronic inflammatory diseases such as RA. As well, targeting NF-κB has been shown to be an effective therapy in animal models of arthritis (1).

As discussed above, TRAF1 regulates canonical NF-κB activation downstream of TNFRs and TLRs and multiple genome-wide association studies have found SNPs at the TRAF1-C5 locus (encoding TRAF1 and complement component 5) on chromosome 9 associated with an increased risk for RA (61,70–73). In addition, Cheng et al. compared serum concentrations of TRAF1 in RA patients with healthy individuals and found a significantly higher TRAF1 concentration in RA patients and a positive correlation between autoantibodies and TRAF1 (74). Furthermore, patients with the TRAF1-C5 SNP rs3761847 GG homozygote status were at an increased risk of death from sepsis and malignancies in RA patients (75). Lastly, both monocytes and T-cells from healthy subjects with a disease-associated SNP expressed lower levels of TRAF1 and this was associated with increased inflammation (69).

Hence, TRAF1 may be a possible target for therapeutic intervention in treating RA as it may play a crucial role in the pathophysiology of RA, however, more research needs to be conducted regarding the exact mechanisms.

The opposing effects of TRAF1 are both important regarding the immune response, whereby activation of NF-κB, induces production of pro-inflammatory cytokines and chemokines crucial for the clearance of infections while inhibiting NF-κB overactivation is essential in preventing chronic inflammation as seen in rheumatoid arthritis (RA) and other autoimmune diseases (76). This also makes it very difficult to understand its role in a complex autoimmune disease such as RA, as Cheng et al. found no effect of TRAF1 in an animal model of inflammatory arthritis, likely due to the opposing effects of TRAF1 (76).

2.0 Rationale and Objectives

2.1 Rationale

The overwhelming evidence suggests TRAF1 may be involved in the pathophysiology of RA. However, TRAF1 plays opposing roles in different cell types and receptors. In lymphocytes, downstream of 4-1BB in T cells and CD40 in B cells, it positively regulates NF-κB activity, through the cIAP2 axis; while in monocytes/macrophages downstream of TLR signaling, TRAF1 inhibits NF-κB activation by interfering with LUBAC. Therefore, understanding TRAF1's role in RA requires dissecting these opposing effects on NF-κB activation and studying them individually in-vivo, which could eventually lead to a TRAF1-target therapy that provides positive outcomes in RA patients. Dissecting these opposing effects requires understanding of the exact protein-protein interaction between TRAF1 and these molecules.

2.2 Objectives

1) Identify the sites of interaction between TRAF1 and LUBAC components
2) Create a TRAF1 mutant in which the interaction between LUBAC components is abrogated but maintained with cIAP2 and TRAF2
3) Verify the TRAF1 mutant positively regulates NF-κB activation in vitro through a functional assay

2.3 Hypothesis

We hypothesize the specific protein-protein interaction between TRAF1 and LUBAC components occurs in the MATH domain. We also hypothesize the TRAF1 mutant we generate will thwart the interaction between LUBAC components while maintaining the interaction with cIAP2. Functionally, this TRAF1 mutant will positively regulate NF-κB activity downstream of TNFR family members but with its ability to inhibit NF-κB activity downstream of TLRs abrogated.

3.0 Methods

3.1 Cell Culture

293 T/17 cells were cultured in T75 flasks at 37°C, 20% O_2 and 5% CO_2, in DMEM (SIGMA), supplemented with 10% fetal bovine serum (FBS) (SIGMA) and 1% L-Glutamine-Penicillin-Streptomycin (GPS). Media was replenished every 48 hours and cells were grown to 70% confluence before being trypsinized with 5 mL of trypsin (Gibco) for 5 minutes at 37°C. Trypsinized cells were added to an equal volume of media and centrifuged at 1500 RPM for 5 minutes at 4°C. Following centrifugation, media was removed and cells were resuspended in fresh media.

3.2 Plasmids

Human TRAF1 coding sequence, as well as, truncations: TRAF1-ΔMATH, TRAF1-ΔMATHCC and TRAF1-MATH+CC were cloned into the c-FLAG pcDNA3 vector. pCMV3flag8SHARPIN, pCMV3flag8HOIP and pCMV3flag8 HOIL-1L were gifts from Martin Dorf (Addgene plasmid # 50014, 50015 and 50016, respectively) (77).

3.3 Bacterial Culture

3.3.1 Transformation

Initially, all plasmids were transformed into DH5-α bacteria by incubating 100 ng of each plasmid with 100 μL of DH5-α cells for 30 mins on ice. Following incubation, bacteria were heat-shocked at 42°C for 45 seconds. Subsequently, they were left to incubate on ice for 2 mins before 1 mL of LB broth was added. The bacteria were placed in an orbital shaker for 1 hour at 37°C and 250 RPM. Afterwards, 100 μL of the bacterial mixture was spread on a LB agar plate with ampicillin resistance and left to incubate at 37°C overnight.

3.3.2 Plasmid Miniprep

A colony was picked from the agar plate with a sterile pipette tip and left to incubate in 3 mL of LB Broth containing ampicillin overnight in an orbital shaker at 37°C and 250 RPM. The following day, with bacterial growth present, glycerol stocks were prepared by adding 850 µL of the overnight bacterial culture to 150 µL of 100% glycerol which were then stored at -80°C. The rest of the culture was used for DNA plasmid isolation using the GenElute HP Plasmid Miniprep kit (Sigma).

3.3.3 Plasmid Midiprep

For higher DNA plasmid yields, a starter culture was prepared for 6-8 hours using the exact same protocol as used for the overnight bacterial culture. After incubation, 100 µL of the starter culture was added to 75 mL of LB broth and left overnight to incubate in an orbital shaker at 37°C and 250 RPM. The following day, with bacterial growth present, plasmid DNA was isolated form bacterial culture using the GenElute HP Plasmid Midiprep kit (Sigma). Recently, we have switched to the Midiprep kit (Thermo), which follows a similar protocol.

3.4 Cloning

In order to create the TRAF1 truncated derivatives, we implemented Polymerase Chain Reaction (PCR) and Megaprimer PCR. For the following truncations: TRAF1-ΔAA400-416, TRAF1-ΔAA314-416, TRAF1-ΔAA289-416 and TRAF1-ΔAA277-416 we used regular PCR. The forward primer was the same for each, consisting of a XbaI site and the first 18 base pairs of the TRAF1 gene. However, the reverse primers differed, containing a BamHI restriction site and the last 18 complementary base pairs before the desired deletion of the MATH domain.

For the TRAF1 truncations in which we deleted select 17 AA regions of the MATH domain, except for TRAF1-ΔAA400-416, we used Megaprimer PCR, which required 3 different primers and 2 rounds of PCR amplification to create the desired truncation. The first PCR reaction

created the reverse primer for the second PCR reaction; 2 of the 3 primers for each truncation were the exact same, TRAF1 XBaI Forward and Mega Reverse primers, as they flanked the ends of the TRAF1 gene and the third provided the desired mutation.

3.4.1 PCR Amplification

The first step in creating these TRAF1 truncated derivatives was to amplify the PCR product. Human TRAF1 coding DNA sequence cloned into a c-Flag pcDNA3 vector was used as the template. Table 1 shows the amount of each reagent used and Table 2 provides the PCR conditions. Table 3 and Table 4 show the amount of each reagent used and the PCR conditions for the megaprimer second PCR, respectively. Following each PCR amplification, the PCR product was run on a 1% Agarose gel and then extracted from the gel using the GenElute Gel Extraction Kit (Sigma).

Table 1. Cloning PCR Reaction

	50 µL Reaction	Final Concentration
dNTP Mix	2.5 µL	500 µM
10X Accu Taq Buffer	5 µL	1X
Accu Taq DNA Polymerase	0.5 µL	0.05 u
10 µM Forward Primer	2.0 µL	0.4 µM
10 µM Reverse Primer	2.0 µL	0.4 µM
TRAF1-FL (500 ng/µL)	1 µL	500 ng
MBG H_2O	37 µL	

Table 2. Cloning PCR Conditions

Step	Temperature	Time	
Initial Denaturation	96°C	30 seconds	
25 Cycles	94°C	10 seconds	
	62°C	30 seconds	
	68°C	90 seconds	
Final Extension	68°C	15 min	
Hold	4°C	Infinite Hold	

Table 3. Megaprimer Second PCR Reaction

	50 µL Reaction	Final Concentration
dNTP Mix	2.5 µL	500 µM
10X Accu Taq Buffer	5 µL	1X
Accu Taq Polymerase	0.5 µL	0.05 u
10 µM TRAF1XbaI Forward Primer	2.0 µL	0.4 µM
1st PCR Product	2.5 µL	0.4 µM
TRAF1-FL (500 ng/µL)	1 µL	500 ng
MBG H_2O	36.5 µL	

Table 4. Megaprimer Second PCR Conditions

Step	Temperature	Time	
Initial Denaturation	96°C	120 seconds	
25 Cycles	94°C	30 seconds	
	58°C	30 seconds	
	68°C	90 seconds	
Final Extension	64°C	5 min	
Hold	4°C	Infinite Hold	

3.4.2 Restriction Enzyme Digestion

The recovered and purified PCR product and c-Flag pcDNA3 vector were restriction enzyme (RE) digested with BamHI and XbaI for an hour at 37°C and subsequently purified using the GenEltute PCR Clean-Up Kit (Sigma). In this reaction, 5 µL of either the PCR product or vector was used and to it 1 µL each of BamHI (NEB) and XbaI (NEB), 1 µL of 10X Cutsmart Buffer (NEB) and 38 µL of MBG H_2O were added.

3.4.3 Ligation and Transformation

The purified, RE digested PCR product was ligated into the purified, RE digested c-Flag pcDNA. 65 ng of the vector was ligated with a select amount of insert calculated using the Insilico ligation calculator at a 3:1 vector:insert ratio. In this reaction, 1 µL of T4 DNA Ligase (NEB) and 4 µL of the 5X Ligase Buffer (NEB) were added to the respective amounts of vector and insert and the corresponding amount of MBG H_2O to raise the volume to 20 µL. The reaction was left to incubate at room temperature for 4 hours and then transformed into DH5-α bacteria, using the protocol described above.

3.5 Site-directed Mutagenesis

The Q5 Site-Directed Mutagenesis Kit (NEB) was used to produce TRAF1 truncations, TRAF1-ΔAA266-341, TRAF1-ΔAA339-412, TRAF1-ΔCC and single AA substitutions of TRAF1. Human TRAF1 coding DNA sequence cloned into a c-Flag pcDNA3 vector was used as the parent plasmid to which site-directed mutagenesis was performed. Primers were designed using the NEBaseChanger tool and ordered from IDT.

3.5.1 PCR Amplification

The first step in this kit was amplification of the PCR product. Table 5. provides the amount of each reagent used and Table 6. provides the cycling conditions. Note annealing temperature was adjusted to Ta of primers. In addition, final extension was extended from 2 minutes to 5 minutes.

Table 5. SDM PCR reaction

	25 µL Reaction	Final Concentration
Q5 Hot-Start High Fidelity 2X Master Mix	12.5 µL	1X
10 µM Forward Primer	1.25 µL	0.5 µM
10 µM Reverse Primer	1.25 µL	0.5 µM
TRAF1-FL (10 ng/µL)	1 µL	10 ng
MBG H_2O	9 µL	

Table 6. SDM PCR conditions

Step	Temperature	Time	
Initial Denaturation	98°C	30 seconds	
25 Cycles	98°C	10 seconds	
	50-72°C	30 seconds	
	72°C	30 seconds	
Final Extension	72°C	5 min	
Hold	4°C	Infinite Hold	

3.5.2 KLD Reaction

The second step was to mix 1 µL of the PCR product with 5 µL of 2X KLD reaction buffer, 10X KLD enzyme mix and 3 µL of molecular biology grade (MBG) H_2O and incubate the mixture for 45 minutes at room temperature. Note incubation time was changed from 5 minutes to 45 minutes.

3.5.3 Transformation

The last step was the transformation, in which 5 µL of the KLD reaction was mixed with 50 µL of bacterial cells and incubated on ice for 30 minutes. The bacteria were heat-shocked at 42°C for 30 seconds and left to incubate on ice for an additional 5 minutes. 950 µL of SOC broth was added to the bacteria and they were incubated at 37°C for 1 hour at 250 RPM in an orbital shaker. 100 ul of mixture was spread on a LB agar ampicillin plate and left to incubate overnight at 37°C.

3.6 DNA Transfection

HEK 293 T/17 cells were trypsinized in 5 mL of trypsin for 5 minutes at 37°C and 1.5×10^6 cells were seeded in 6-cm plates overnight. Cells were transfected with 2 μg of either HOIL1 or HOIP and TRAF1 full length (FL) or truncation/mutant. As controls, HOIL1, HOIP and TRAF1 FL were co-transfected with the c-Flag pcDNA3 vector. On the day of transfection, media was replaced with 3.3 mL of Opti-MEM (Gibco). DNA mixes, comprised of 600 μL of Opti-MEM, 2 ug of each plasmid and 8 μL of P3000 (Invitrogen) and Lipofectamine mixes, comprised of 600 μL of Opti MEM and 8 μL LIPO 3000 (Invitrogen), were mixed and left to incubate for 15 minutes at room temperature before being added onto cells. Cells were left to incubate for 6 hours before media was changed to full-growth.

3.7 Coimmunoprecipitation

Cells were lysed with 500 μL of Co-IP lysis buffer (20 mM Tris-HCl pH 8, 137 mM NaCl, 10% glycerol, 1% NP-40, 2 mM EDTA) containing Complete Protease Inhibitor Cocktail, PhosSTOP Phosphatase Inhibitor Cocktail and 1 M DTT (Sigma).
Before performing Co-IP, 15 μL of lysate was removed and used for input. To the lysates 0.75 μg of TRAF1 antibody (clone 1F3) was added and incubated for 15 minutes on a rotator (speed 21) at room temperature. Each Lysate was then incubated with 25 μL Protein G Dynabeads (Invitrogen), pre-washed with PBS + 0.02% Tween, for 15 minutes on a rotator (speed 21) at room temperature. Beads were then washed with Co-IP lysis buffer, containing no inhibitors, 3 times and with PBS 2 times before being eluted with 25 μL of 2X Laemmeli Buffer (BioRad).

3.8 Immunoblotting

Immunoprecipitated proteins were heated at 95-100°C for 5 minutes and loaded onto 10% polyacrylamide gels, ran at 60 V for 30 minus and at 120 V for 80 minutes and then transferred to PVDF membranes. Membranes were blocked for 1 hour in 5% skim milk and incubated with

respective primary antibody (1:1000 dilution) at 4°C overnight. The following day, membranes were washed with TBST 3 times for 10 minutes, incubated with secondary antibody diluted in 10 mL of 5% skim milk (1:10000 dilution), and subsequently washed 3 more times with TBST for 10 minutes. Blots were imaged using Clarity ECL (BioRad).

3.9 Crispr/Cas9 mediated gene editing

TrueGuide crRNA (Invitrogen) were designed to target sequences of the TRAF1 locus with maximum knock-out efficiency while minimizing off-target effects. A total of 3 TrueGuide crRNA were selected, one from Invitrogen's CRISPR design tool, one from IDT's CRISPR design tool and one using the CRISPOR program. TrueGuide crRNA and TrueGuide tracrRNA (Invitrogen) were annealed using the manufacturer's protocol. THP-1 cells (0.5×10^6) were seeded in 24-well plates overnight. Cells were transfected with the crRNA:tracrRNA duplex, TrueCut Cas9 Protein v2 (Invitrogen) and Lipofectamine Cas9 Plus Reagent (Invitrogen). Day of transfection, media was replaced with 450 µL of Opti-MEM. DNA mixes, comprised of 25 µL of Opti-MEM, 0.4 µL of TrueCut Cas9 Protein v2, 1.2 µL of the crRNA:tracrRNA duplex and 4 µL of Lipofectamine Cas9 Plus Reagent were made. Lipofectamine CRISPRMAX Reagent (Invitrogen) was diluted by adding 1.5 µL of Lipofectamine CRISPRMAX Reagent to 25 µL of Opti-MEM. The diluted Lipofectamine CRISPRMAX Reagent was added to DNA mixes and left to incubate at room temperature for 15 minutes and then added onto cells. After 2 days, media was replaced with full-growth media. The GeneArt Genomic Clevage Detection Kit (Invitrogen) was used on a portion of the cells to verify gene editing efficiency while the rest of the cells were left to grow.

Table 7. List of Primers used for each TRAF1 truncation/mutant and respective Ta

TRAF1 Truncation/ Mutant	**Forward Primer (5'-3')**	**Reverse Primer (5'-3')**	**Annealing Temperature (Ta)**
TRAF1-ΔAA400-416	***TRAF1 XBaI Forward Primer:*** -ATATCTAGAACCATGG CCTCCAGCTCAGGC	-ATAGGATCCGGCG TGCTTGGGTGACTG	62°C
TRAF1-ΔAA383-399	-TRAF1 XBaI Forward Primer -AACCAACGTGGCCAGT TACGTGAAGGACGACA	***Mega Reverse Primer:*** -ATAGGATCCAGTGC TGGTCTCCACAAT	62°C 58°C
TRAF1-ΔAA366-382	-TRAF1 XBaI Forward Primer -CGCCTTCCGGCCTGACG ATGCCCACTCTTTCT	-Mega Reverse Primer	62°C 58°C
TRAF1-ΔAA349-365	-TRAF1 XBaI Forward Primer -CGTGATCATGAGAGGG CTGCTGGACCAGAACA	-Mega Reverse Primer	62°C 58°C
TRAF1-ΔAA332-348	-TRAF1 XBaI Forward Primer -CGTGATCATGAGAGGG CTGCTGGACCAGAACA	-Mega Reverse Primer	62°C 58°C
TRAF1-ΔAA315-331	-TRAF1 XBaI Forward Primer -GTACCTGAATGGAGAT GAGTATGATGCGCTGC	-Mega Reverse Primer	62°C 58°C
TRAF1-ΔAA298-314	-TRAF1 XBaI Forward Primer -CTCCCCAGCCTTCTA CGGCACTGGAAAGAGA	-Mega Reverse Primer	62°C 58°C
TRAF1-ΔAA281-297	-TRAF1 XBaI Forward Primer -TGTCACCAGGCGGTGC ACTGCCAAGTATGGT	-Mega Reverse Primer	62°C 58°C
TRAF1-ΔCC	-TTCGATGGCACTTTCCT GTG	-GAAGTGCTGCAGGG CCAG	66°C
TRAF1-ΔAA266-341	-CGGAACAAGGTCACCT TC	-GAAGGAGGCCTCCT CCAT	63°C
TRAF1-ΔAA339-412	-GAGACCAGCACTGGAT CC	-CGGCAGCAGCGCAT CATA	66°C

TRAF1-ΔAA314-416	-TRAF1 XBaI Forward Primer	-ATAGGATCCTCCATTCAGGTACAGCCG	62°C
TRAF1-ΔAA289-416	-TRAF1 XBaI Forward Primer	-ATAGGATCCGGTCCTGCCACAGGCCGA	62°C
TRAF1-ΔAA277-416	-TRAF1 XBaI Forward Primer	-ATAGGATCCGACATTGGTGATCTTCCA	62°C
TRAF1-T277A	-CACCAATGTCGCCAGGCGGTG	-ATCTTCCACAGGAAAGTGCCATC	68°C
TRAF1-R278A	-CAATGTCACCGCGCGGTGCCATG	-GTGATCTTCCACAGGAAAG	60°C
TRAF1-R279A	-TGTCACCAGGGCGTGCCATGAG	-TTGGTGATCTTCCACAGG	61°C
TRAF1-C280A	-CACCAGGCGGGCCCATGAGTCG	-ACATTGGTGATCTTCCACAG	62°C
TRAF1-H281A	-CAGGCGGTGCGCTGAGTCGGCC	-GTGACATTGGTGATCTTCCAC	64°C
TRAF1-E282A	-CGGTGCCATGCGTCGGCCTGT	-CCTGGTGACATTGGTGATCTTCC	68°C
TRAF1-S283A	-GTGCCATGAGGCGGCTGTGG	-CGCCTGGTGACATTGGTGATCTTC	71°C
TRAF1-C285A	-TGAGTCGGCCGCTGGCAGGACCGTCAGC	-TGGCACCGCCTGGTGACA	70°C
TRAF1-G286A	-TCGGCCTGTGcCAGGACCGTC	-CTCATGGCACCGCCTGGT	71°C
TRAF1-R287A	-GGCCTGTGGCGCGACCGTCAGC	-GACTCATGGCACCGCCTG	66°C
TRAF1-T288A	-CTGTGGCAGGGCCG TCAGCCT	-GCCGACTCATGGCACCGC	72°C

4.0 Results

Effect of TRAF1-ΔMATH, TRAF1-ΔMATH/CC and TRAF1-MATH+CC on the interaction with HOIL1

TRAF1 is known to interact with all 3 components of LUBAC, HOIL1, HOIP and SHARPIN, through its MATH domain, amino acid residues 266-412. This was shown by direct interaction of purified proteins using a GST pull down assay (75). Since we are employing an overexpression system in HEK 293T/17 cells, the first step was to verify, that TRAF1, indeed, interacts with LUBAC through its MATH domain, before targeting specific sites of the MATH domain for interaction. We generated 3 different truncations of TRAF1: TRAF1-ΔMATH, deletion of only the MATH domain; TRAF1-ΔMATH/CC, deletion of the Coiled-Coil (CC) and MATH domain and TRAF1-MATH+CC, consisting of only the CC and MATH domain.

LUBAC consists of the aforementioned 3 components, examining how the interaction is affected between each TRAF1 truncation and each LUBAC component was not ideal. Since both HOIL1 and HOIP interact with TRAF1 through their NZF domains, it is very likely both interact at the same site of TRAF1. Hence, we decided to determine the sites of interaction with HOIL1 first. We overexpressed TRAF1 full length (FL) and truncations, in addition, to HOIL1 in 293T/17 cells, followed by co-immunoprecipitation of lysates with a TRAF1-specfic antibody and immunoblotted for HOIL1. As negative controls, we overexpressed TRAF1 and HOIL1 on their own, co-transfected with the empty plasmid (c-Flag pcDNA3 vector) to maintain the same amount of total DNA transfected.

Figure 5 shows a Western Blot of an immunoprecipitation conducted with a TRAF1-specific antibody and although the interaction between TRAF1 and HOIL1 is completely abrogated with TRAF1 -ΔMATH and -ΔMATH/CC, it is also abolished with TRAF1-MATH+CC, which consists of both the MATH and CC domains alone. The MATH domain is

known to be the site of interaction between LUBAC components; hence, these results suggest either the interaction between TRAF1 and HOIL1 occurs at multiples sites of the protein or the TRAF1 truncation is not being immunoprecipitated because the TRAF1-specific antibody binds to a portion of TRAF1 that is deleted i.e. somewhere between amino acid residues 1-216. This led us to perform an immunoprecipitation with a FLAG-specific antibody.

Comparison between an immunoprecipitation performed with a TRAF1-specific antibody and FLAG-specific antibody

The FLAG tag is an 8-AA peptide (DYKDDDDK) which can be added to the N- or C-terminus of a target protein to improve purification or detection as it is small and hydrophilic, so it resides on the surface and does not affect folding of the protein (68). Since TRAF1 FL and the truncated derivatives were cloned into the c-FLAG pcDNA3 vector, they contained a C-terminus FLAG tag. Therefore, we conducted an immunoprecipitation with both the TRAF1-specific antibody and FLAG-specific antibody to confirm if TRAF1-MATH+CC was, indeed, not immunoprecipitated by the TRAF1-specific antibody. Figure 6 (a) shows TRAF1-MATH+CC is not immunoprecipitated by the TRAF1-specific antibody, however, it is being expressed as seen in the lysate. In contrast, Figure 6 (b) shows TRAF1-MATH+CC is immunoprecipitated by the FLAG-specific antibody. Hence, TRAF1 likely interacts with HOIL1 through its MATH domain.

Effect of TRAF1-ΔMATH and TRAF1-ΔCC on the interaction with HOIL1

The TRAF domain consists of the N-terminus CC domain and the C-terminus MATH domain. Our results showed deletion of either the MATH domain only (TRAF1-ΔMATH) or, the MATH and CC domain together (TRAF1-ΔMATHCC) abolished the interaction between HOIL1. Hence, there was still a possibility the interaction between TRAF1 and HOIL1 occurred at both domains. This led us to construct a TRAF1 where only the CC domain was deleted and the rest of the protein remained intact, TRAF-ΔCC. Figure 7 (a) shows that when only the CC domain is deleted the interaction between TRAF1 and HOIL1 remains intact, but is mitigated

when the MATH domain is deleted. In addition, Figure 7 (b) confirms both TRAF1 mutants are equally immunoprecipitated, thus, it is a truly a loss of interaction and not lack of the TRAF1 mutant that is producing the results. Meaning that CC domain is not required for interaction.

Effect of deleting 17 amino acid residues of the MATH domain on the interaction with HOIL1

After verifying the MATH domain as the site of interaction between HOIL1, we wanted to further narrow down the region of interaction within the MATH domain, hence, we created TRAF1 mutants, with 17 amino acid residues of the MATH domain deleted so that each mutant targeted a distinct portion of the domain and collectively the mutants targeted the entire domain. Figure 8 shows the co-immunoprecipitation between these mutants and HOIL1 and the results show each mutant interacts with HOIL1 equally when compared to the positive control, TRAF1 FL, which was very surprising. Although we were unsuccessful in cloning TRAF1-ΔAA366-382 and performing the co-immunoprecipitation with this mutant, we hypothesized the interaction might lie at multiple sites of the MATH domain and targeting only 17 amino acid residues might be too small of a region. In addition, by deleting a portion of the protein and combining the distinct ends together we might have altered the folding of the protein, possibly, creating a binding site for HOIL1.

Effect of TRAF1-ΔAA266-341 and TRAF1-ΔAA339-412 on the interaction with HOIL1

Our next plan was to target a larger portion of the MATH domain so we generated TRAF1 truncations with each half of the MATH domain deleted: TRAF1-ΔAA266-341 and TRAF1-ΔAA339-412. These 2 truncations, along with TRAF1-ΔMATH and TRAF1 FL were each co-transfected with HOIL1 followed by co-immunoprecipitation with a TRAF1-specific antibody and immunoblotted for HOIL1 and TRAF1. Figure 9 (a) shows a decrease in the

interaction between TRAF1-ΔAA266-341 and HOIL1 when compared to the positive control, TRAF1 FL, however, this is not seen between TRAF1 ΔAA339-412. Once again, each TRAF1 truncation is equally immunoprecipitated, Figure 9 (b). These results suggest the interaction between TRAF1 and HOIL1 occurs closer to the N-terminus of the MATH domain.

Effect of TRAF1-ΔAA289-416 and TRAF1-ΔAA314-416 on the interaction with HOIL1

We predicted if we continued to truncate the MATH domain, we would eventually delete enough of the domain to completely abrogate the interaction between TRAF1 and HOIL1. Thus, we created the TRAF1 truncations: TRAF1-ΔAA314-416, only 48 amino acid residues of the MATH domain remained, and TRAF1-ΔAA289-416, only 23 amino acid residues of the MATH domain remained. Once again, these truncations along with TRAF1-ΔMATH and TRAF1-FL were co-transfected with HOIL1 followed by co-immunoprecipitation with a TRAF1-specific antibody and immunoblotted for HOIL1 and TRAF1. Figure 10 (a) shows absolutely no change in interaction between these truncations and HOIL1 when compared to the positive control, TRAF1 FL. Therefore, suggesting the MATH domain might need to be further truncated to eradicate the interaction.

Effect of TRAF1-ΔAA277-416 on the interaction with HOIL1

We generated a TRAF1 truncation where only 11 amino acid residues of the MATH domain remained, TRAF1-ΔAA277-416, and along with TRAF1-ΔMATH, TRAF1-ΔAA266-341, TRAF1-ΔAA289-416 and TRAF-FL were co-transfected with HOIL1 followed by co-immunoprecipitation with a TRAF1-specific antibody and immunoblotted for HOIL1 and TRAF1. Figure 11 (a) shows the interaction between HOIL1 is mitigated with TRAF1-ΔAA277-416 to a similar degree as TRAF1-ΔMATH. However, the next smallest truncation, TRAF1-ΔAA289-416, shows the interaction remains completely intact, thus, suggesting the interaction likely lies between residues 277-288.

By narrowing down the site of interaction between HOIL1 and TRAF1 to the first 12 amino acid residues of the MATH domain, it allowed us to move on to the next step which was to individually substitute each residue with alanine to determine specific residue(s) involved in the interaction. Alanine is the first choice substitution that is universally-accepted when assessing protein-protein interactions because it has a side chain that is non-reactive and not bulky, but still mimics the secondary structure preferences that many of the other amino acids possess and does not illicit any electrostatic or steric effects (79). Figure 4 shows the 12 potential amino acids involved in the interaction between TRAF1 and HOIL1. Luckily, of the 12 amino acids residues, 284 was an alanine, so it required us to generate only 11 different mutants.

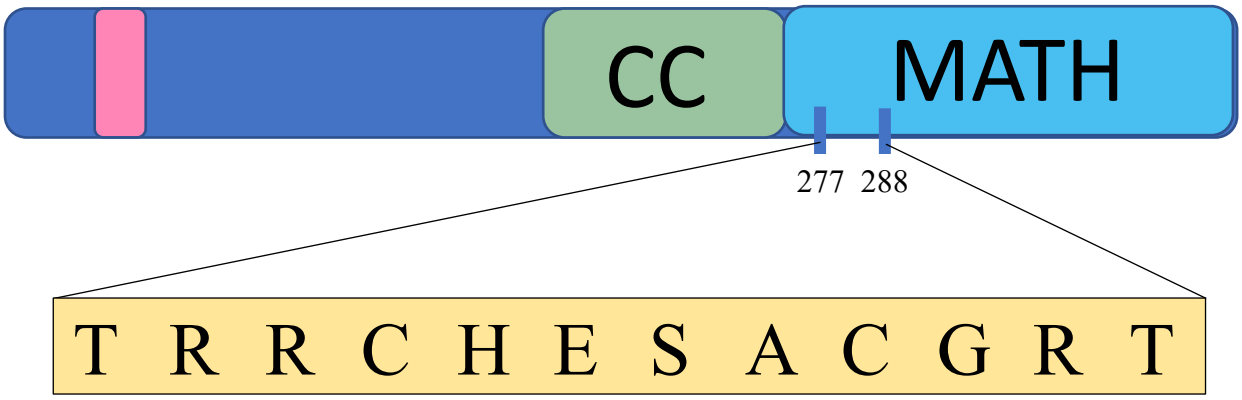


Figure 4. The 12 AA site of interaction between HOIL1 and TRAF1 and their respective one letter codes.

Effect of Single AA Substitutions of TRAF1 Mutants on the interaction with HOIL1

After generating the TRAF1 mutants, we co-transfected each mutant with HOIL1. In addition to having HOIL1 transfected on its own, we used TRAF1-ΔAA277-416 co-transfected with HOIL1 as another negative control. Our positive control was still TRAF FL co-transfected with HOIL1. Following the TRAF1 IP and immunoblotting for HOIL1, Figure 12 (a) shows, once again, the interaction between TRAF1 and HOIL1 is mitigated with TRAF1-ΔAA277-416. Also mutants, TRAF1-H281A and TRAF1-S283A show a discernible decrease in the interaction between HOIL1. Hence, histidine 281 and serine 283 may be potential sites of interaction between HOIL1 and TRAF1. It is important to note we were initially unsuccessful in substituting residues 285 and 288 with alanine, hence, we carried on with experiments and decided to come back to these residues if the other 9 residues did not show interaction.

Effect of TRAF1-H281A and TRAF1-S283 Mutants on the interaction with HOIP

LUBAC is comprised of 3 components, 2 of which are catalytic and of the 2 the main subunit involved in synthesizing linear ubiquitin chains is HOIP. The next step for the project was to examine whether residues 281 and 283 might be sites of interaction between HOIP and TRAF1, as well. Thus, TRAF1 FL, TRAF1-ΔAA277-416, TRAF1-H281A and TRAF1-S283A were co-transfected with HOIP and immunoprecipitated with a TRAF1-specific antibody and immunoblotted for HOIL1 and TRAF1. Figure 13 (a) shows although the interaction is mitigated with TRAF1-ΔAA277-416, it is maintained with both TRAF1-H281A and TRAF1-S283A mutants. Therefore, suggesting the interaction between HOIP and TRAF1 lies in the same region as HOIL1 except with different residues.

Effect of Single AA substitutions of TRAF1 on the interaction with HOIP

Since the interaction between TRAF1 and HOIP occurs between residues 277-288, we next examined how the other TRAF1 mutants, aside from residues 281 and 283, which we had already tested, might affect the interaction between HOIP and TRAF1. Figure 14 (a) shows arginine residue 287 when substituted with an alanine thwarts the interaction between HOIP, almost completely. Threonine residue 288 does show a decrease in interaction, as well, however, this is a result of low expression of HOIP than an actual loss of interaction as evident through the lysate.

Effect of TRAF1-H281E, TRAF1-S283E and TRAF1-H281E/S283E on the interaction with HOIL1

Although we had discerned residues 281 and 283 as sites of interaction with HOIL1, we were informed by a structural biologist that because we were substituting these residues with an alanine, we were possibly disrupting the folding of the protein and by substituting these specific residues with aspartic acid would help maintain the structural integrity of the protein. Hence, I developed TRAF1 mutants where I substituted residues 281 and 283 with an aspartic acid, TRAF1-H281E and TRAF1-S283E, respectively, and a TRAF1 mutant that contained both mutations, TRAF1-H281E/S283E. Along with these mutants, I co-transfected TRAF1-ΔMATH, TRAF1-Δ277-416 and TRAF1-S283A, as negative controls and performed an IP with a TRAF1-speicifc antibody and immunoblotted for HOIL1. Figure 15 (a) shows that when residues 281 and 283 are substituted with an aspartic acid, the effect on the interaction with HOIL1 is of a similar degree to that of TRAF1-S283A, however, collectively, these mutations produce a greater loss in interaction with HOIL1 than on their own and to a similar degree to that of TRAF1-Δ277-416. Therefore, when creating the TRAF1 mutant that does not interact with LUBAC, it might be more effective to substitute residues 281 and 283 with an aspartic acid than an alanine, however, a comparison on how the interaction with HOIL1 is affected between

TRAF1-H281E/S283E and TRAF1-H281A/S283A should be made before reaching this conclusion.

Effect of TRAF1-ΔCC, TRAF1-S283A, TRAF1-R287A and TRAF1-H281E/S283E on the interaction with HOIL1, HOIP and cIAP2

Discerning the specific site(s) of interaction between TRAF1 and LUBAC was the major objective of this project, however, the ultimate, long-term goal is to create a TRAF1 mutant that does not inhibit NF-κB activation and maintains its role as a positive regulator of NF-κB activation through its interaction with cIAP2 and TRAF2. Zheng et al. have shown the interaction between TRAF1 and cIAP2 and TRAF2 occurs in the CC region of TRAF1 (37). In order to corroborate these findings and confirm our potential TRAF1 mutant will maintain interaction with cIAP2 and TRAF2, we co-transfected TRAF1-ΔCC, TRAF1-S283A and TRAF1-H281E/S283E with HOIL1 and cIAP2 and co-transfected the same plasmids with HOIP except replaced TRAF1-S283A with TRAF1-R287A and performed an IP with a TRAF1-speicifc antibody and immunoblotted for the respective proteins. Figure 16 (a) and (c) show TRAF1-ΔCC maintains the interaction with both HOIP and HOIL1 but TRAF1-H281E/S283E thwarts the interaction with both HOIP and HOIL1 when compared to the positive control, TRAF1 FL. In addition, Figure 16 (b) and (d) show TRAF1-H281E/S283E maintains the interaction with TRAF2, as well, but TRAF1-ΔCC does not. Figure 16 (e) shows TRAF1-ΔCC almost completely abolishes the interaction with cIAP2 but the interaction with cIAP2 is maintained with TRAF1-S283A and TRAF1-H281E/283E. Therefore, TRAF1-H281E/283E is a promising candidate to further study as its interaction with HOIL1 and HOIP is diminished but retained with cIAP2 and TRAF2.

5.0 Figures

Figure 5. Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and c-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔMATH, 5) HOIL1 and TRAF1-ΔMATH/CC and 6) HOIL1 and TRAF1-MATH+CC followed by immunoblotting (IB) with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. This experiment was conducted twice.

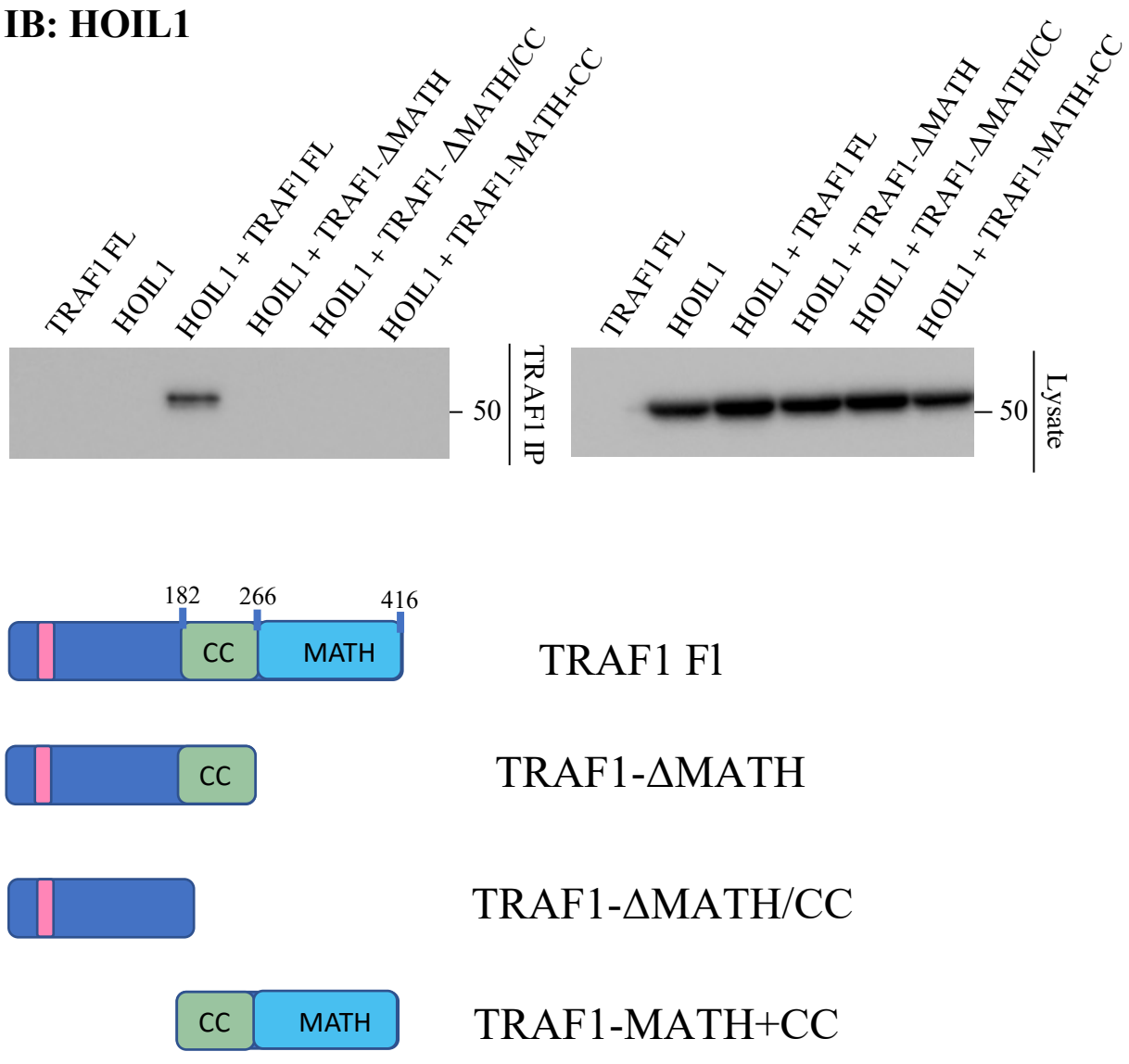


Figure 6. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL, 2) TRAF1-ΔMATH, 3) TRAF1-ΔMATH/CC and 4) TRAF1-MATHH+CC followed by immunoblotting with FLAG-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. (b) Same as (a) but IP performed with FLAG-specific antibody. This experiment was conducted once.

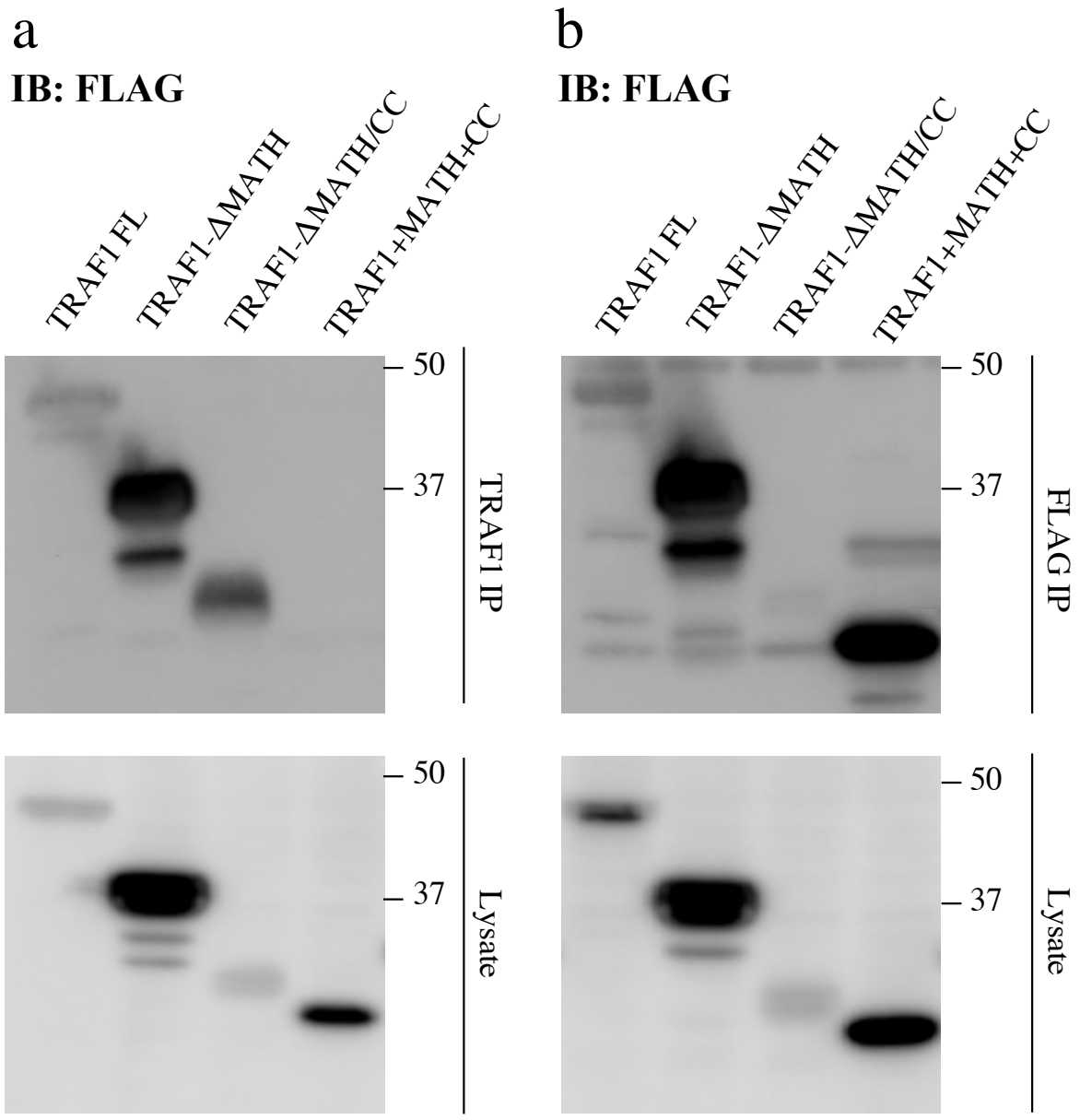


Figure 7. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and C-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔCC and 5) HOIL1 and TRAF1-ΔMATH followed by immunoblotting (IB) with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted twice.

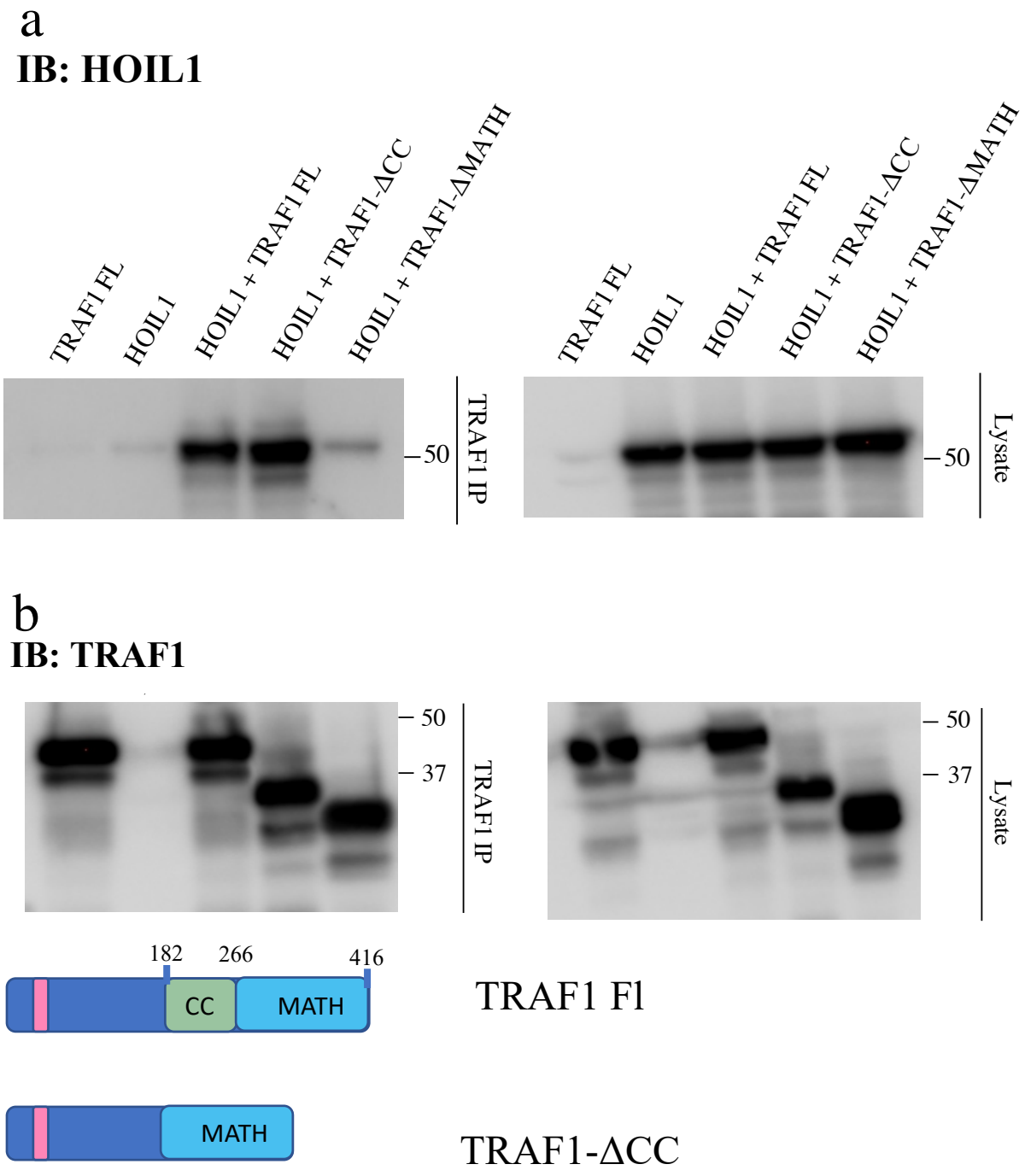
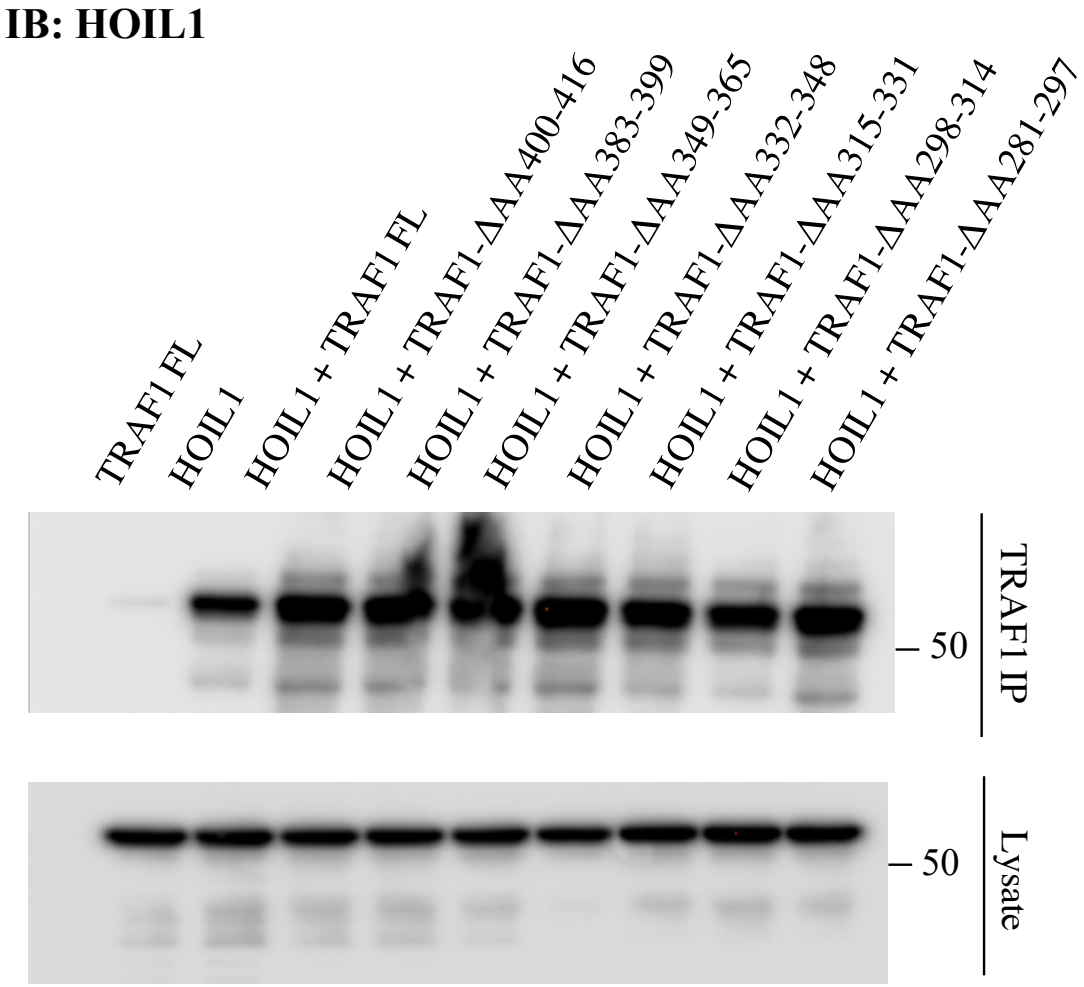
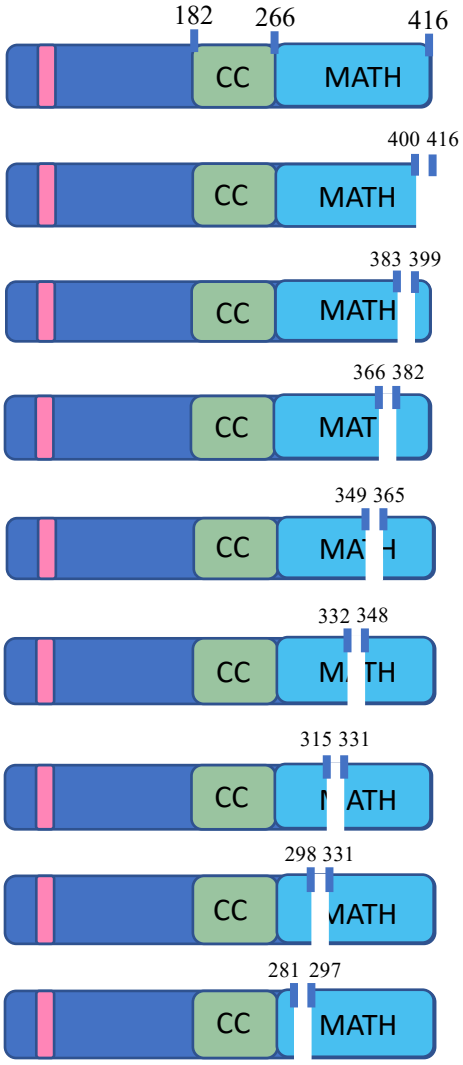


Figure 8. Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and C-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔAA400-416, 5) HOIL1 and TRAF1-ΔAA383-399, 6) HOIL1 and TRAF1-ΔAA349-365, 7) HOIL1 and TRAF1-ΔAA332-348, 8) HOIL1 and TRAF1-ΔAA315-331, 9) HOIL1 and TRAF1-ΔAA298-314 and 10) HOIL1 and TRAF1-ΔAA281-297 followed by immunoblotting with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. This experiment was conducted twice.





TRAF1 Fl

TRAF1-ΔAA400-416

TRAF1-ΔAA383-399

TRAF1-ΔAA366-382

TRAF1-ΔAA349-365

TRAF1-ΔAA332-348

TRAF1-ΔAA315-331

TRAF1-ΔAA298-314

TRAF1-ΔAA281-297

Figure 9. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and c-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔAA266-341, 5) HOIL1 and TRAF1-ΔAA339-412 and 6) HOIL1 and TRAF1-ΔMATH followed by immunoblotting (IB) with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. *Note there was a lane that was removed from this figure, that lane contained HOIL1 and TRAF1-ΔCC; it was removed because the expression of proteins was very low. This experiment was conducted once.

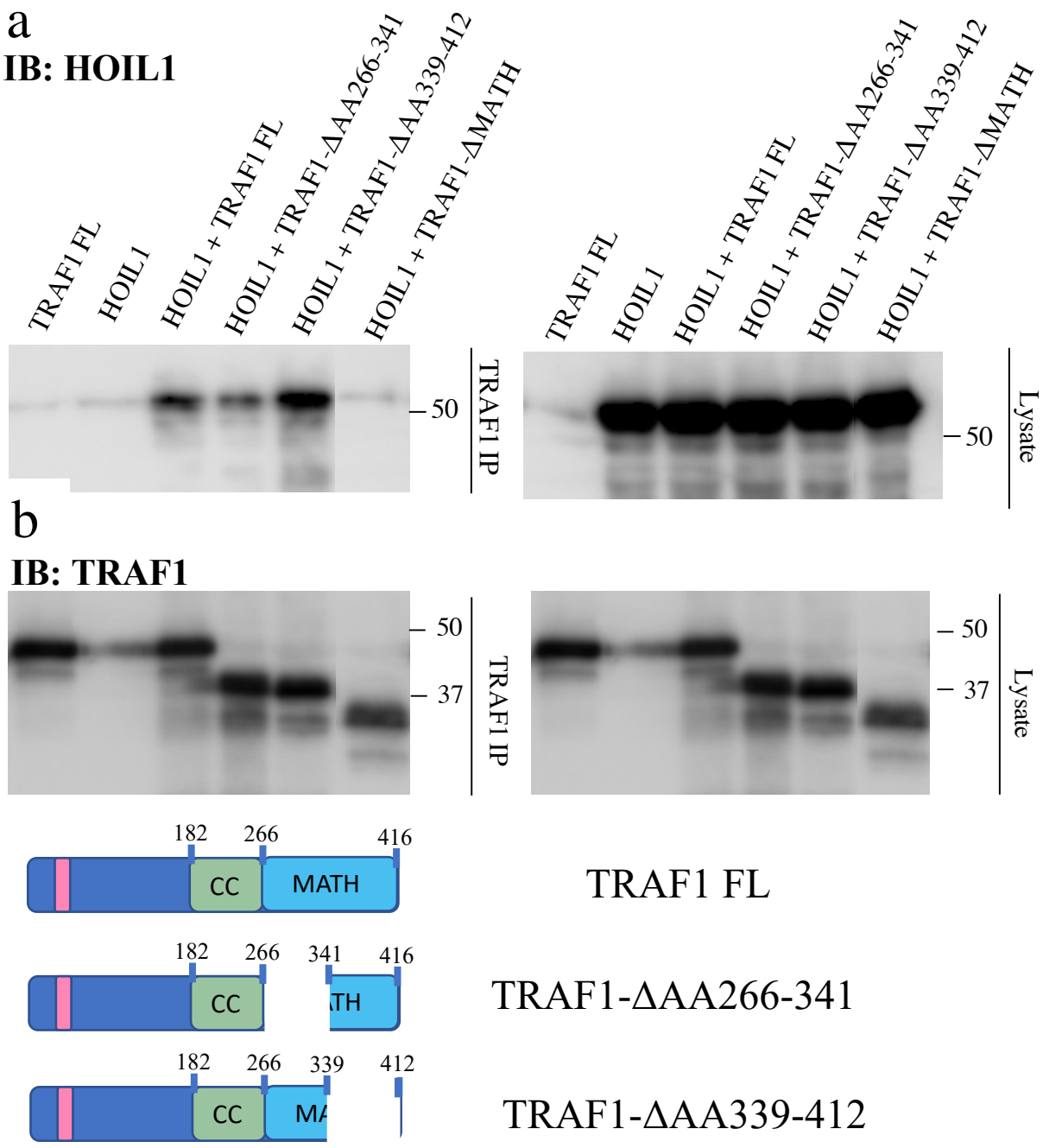


Figure 10. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and c-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔMATH, 5) HOIL1 and TRAF1-ΔAA314-416 and 6) HOIL1 and TRAF1-Δ289-416 followed by immunoblotting (IB) with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted once.

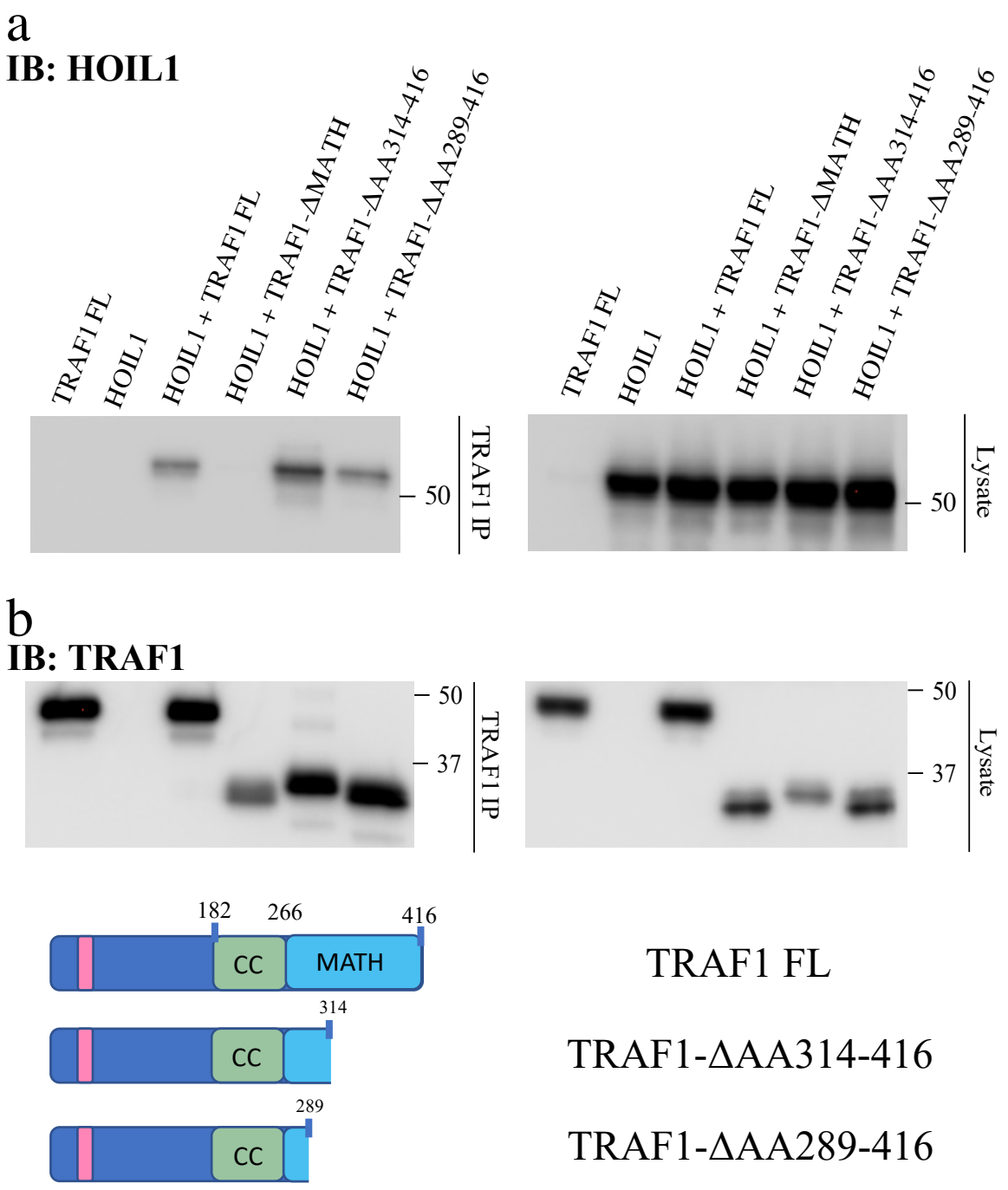


Figure 11. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and c-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1- ΔMATH, 5) HOIL1 and TRAF1-ΔAA266-341, 6) HOIL1 and TRAF1-Δ289-416 and 7) HOIL1 and TRAF1-Δ277-416 followed by immunoblotting (IB) with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted twice.

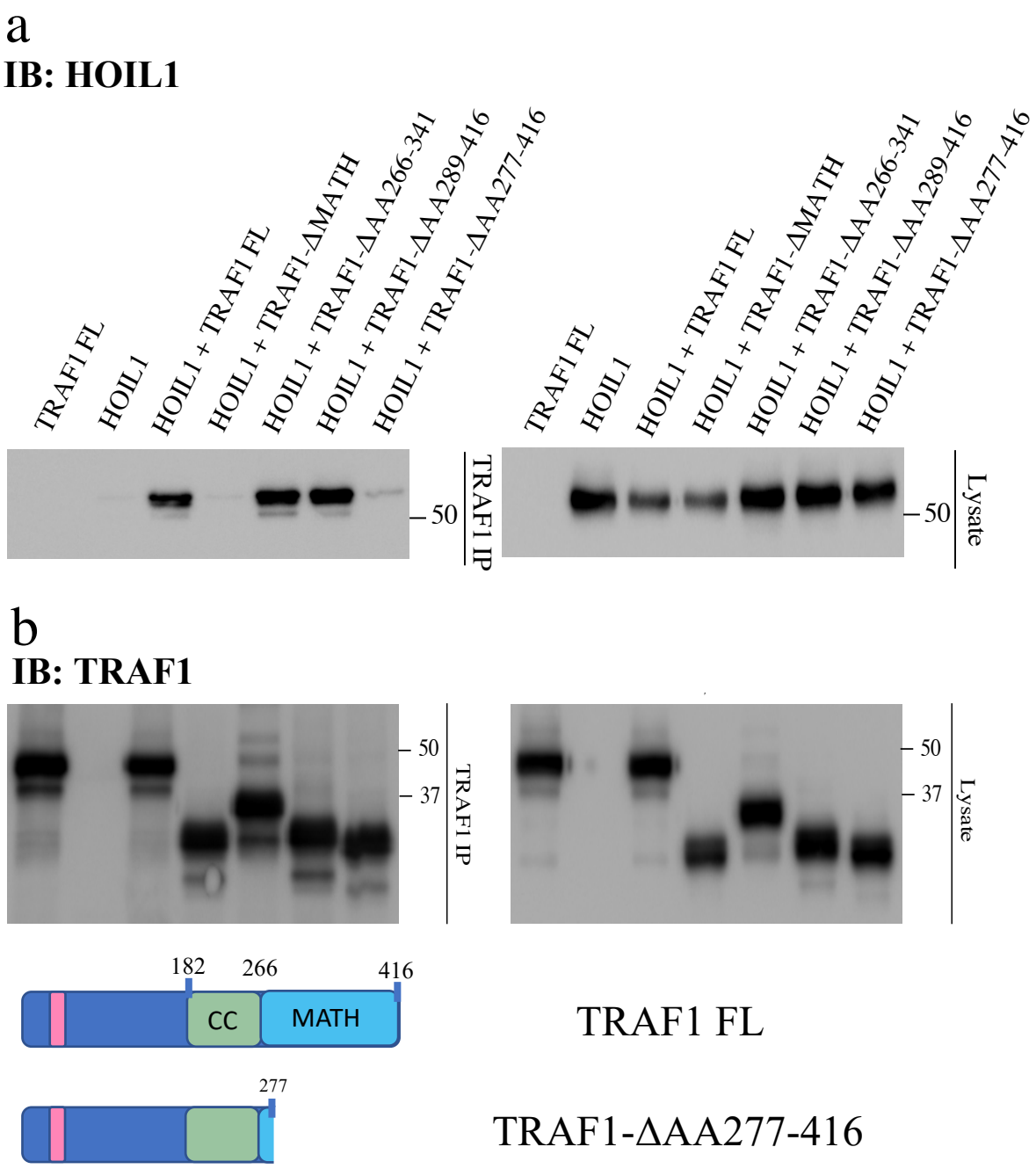


Figure 12. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and c-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔAA277-416, 5) HOIL1 and TRAF1-T277A, 6) HOIL1 and TRAF1-R278A, 7) HOIL1 and TRAF1-R279A, 8) HOIL1 and TRAF1-C280A, 9) HOIL1 and TRAF1-S283A, 10) HOIL1 and TRAF1-G286A, 11) HOIL1 and TRAF1-H281A, 12) HOIL1 and TRAF1-E282A and 13) HOIL1 and TRAF1-R287A followed by immunoblotting (IB) with HOIL1-specfic antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted twice.

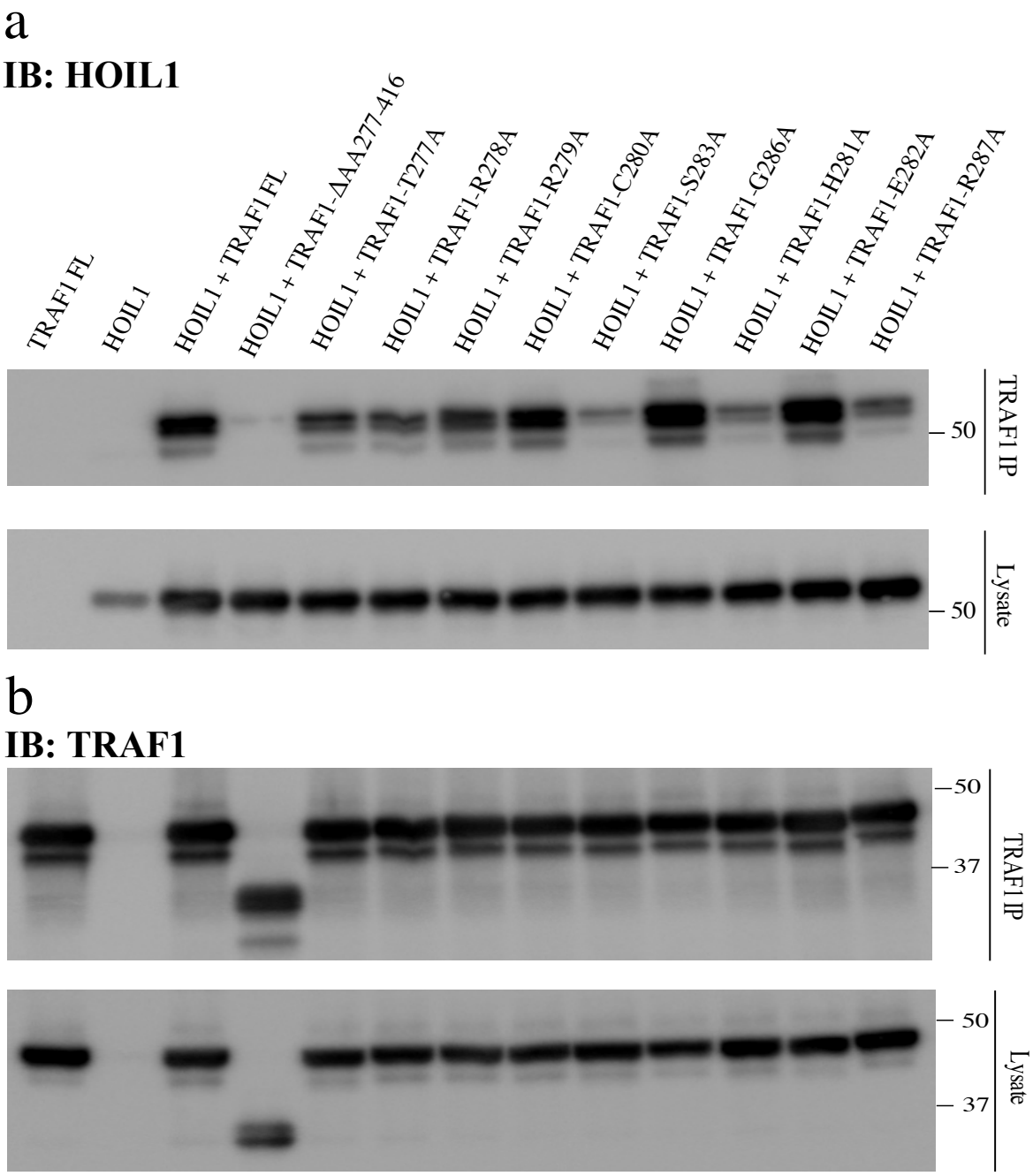


Figure 13. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIP and c-Flag pcDNA3 vector, 3) HOIP and TRAF1 FL, 4) HOIP and TRAF1-ΔAA277-416, 5) HOIP and TRAF1-H281A and 6) HOIP and TRAF1-S283A followed by immunoblotting with HOIP-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted twice.

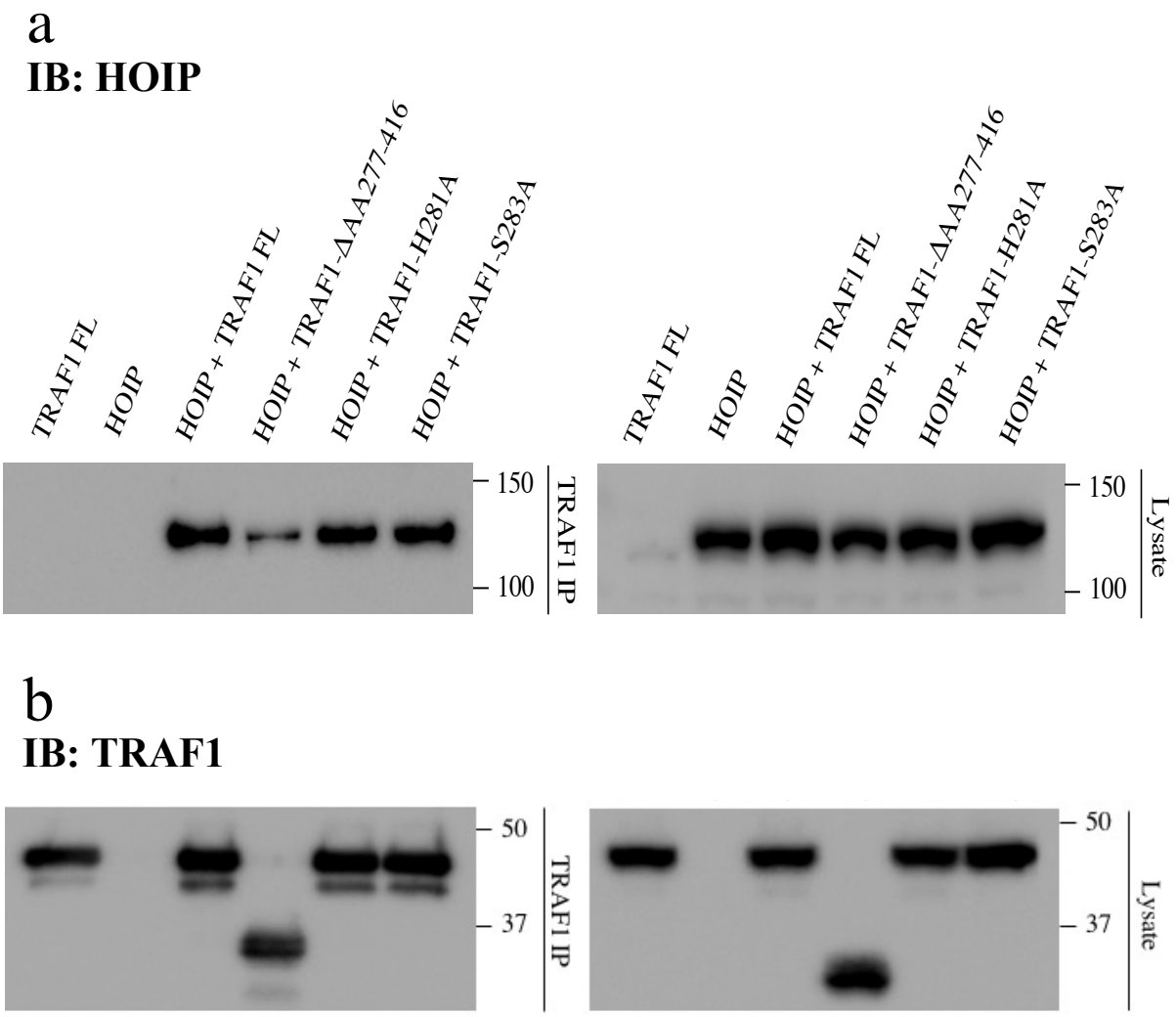


Figure 14. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIP and c-Flag pcDNA3 vector, 3) HOIP and TRAF1 FL, 4) HOIP and TRAF1-ΔAA277-416, 5) HOIP and TRAF1-T277A, 6) HOIP and TRAF1-R278A, 7) HOIP and TRAF1-R279A, 8) HOIP and TRAF1-C280A, 9) HOIP and TRAF1-E282A, 10) HOIP and TRAF1-G286A, 11) HOIP and TRAF1-R287A and 12) HOIP and TRAF1-T288A followed by immunoblotting with HOIP-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted twice.

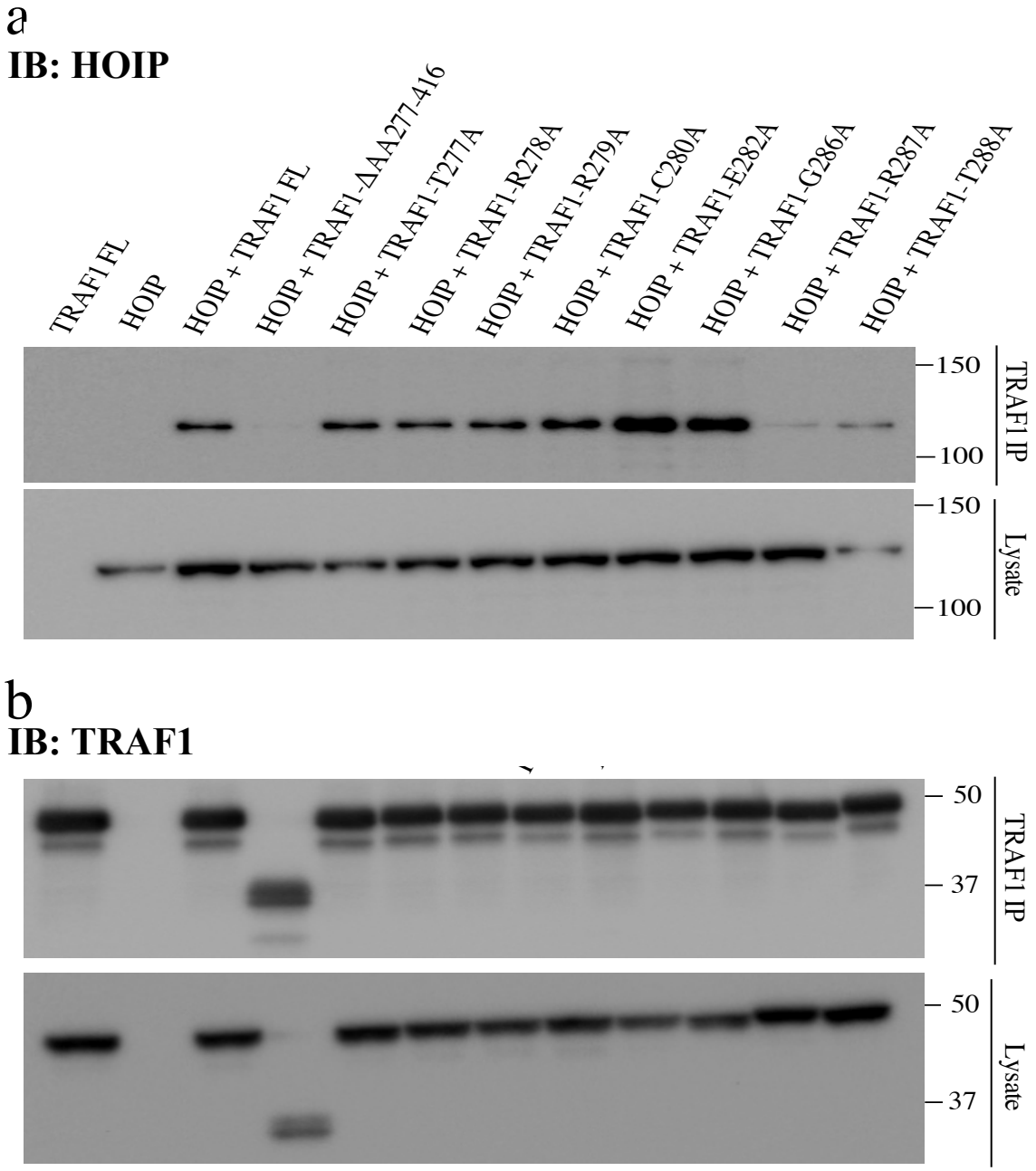


Figure 15. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of lysates of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIL1 and c-Flag pcDNA3 vector, 3) HOIL1 and TRAF1 FL, 4) HOIL1 and TRAF1-ΔMATH, 5) HOIL1 and TRAF1-ΔAA277-416, 6) HOIL1 and TRAF1-S283A, 7) HOIL1 and TRAF1-H281E/S283E 8) HOIL1 and TRAF1-H281E and 9) HOIL1 and TRAF1-S283E followed by immunoblotting with HOIL1-specific antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (b) IB with TRAF1-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted once.

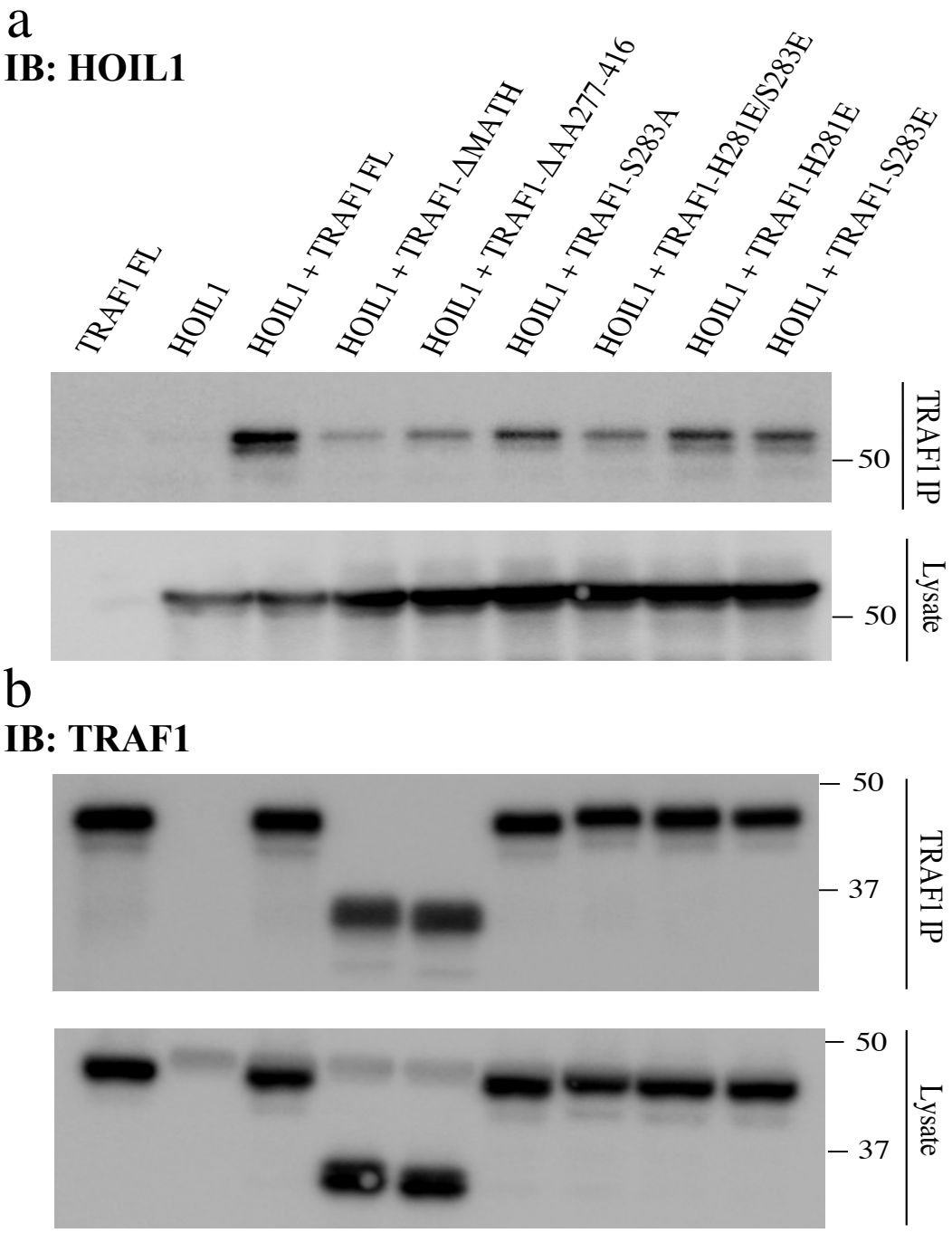


Figure 16. (a) Immunoprecipitation (IP) with TRAF1-specific antibody of HEK 293T/17 cells transfected with 1) TRAF1 FL and c-Flag pcDNA3 vector, 2) HOIP and c-Flag pcDNA3 vector, 3) HOIP and TRAF1 FL, 4) HOIP and TRAF1-ΔCC, 5) HOIP and TRAF1-R287A and 6) HOIP and TRAF1-H281E/S283E followed by IB with HOIP-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. (b) IB with TRAF2-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. (c) IP with TRAF1-specific antibody of HEK 293T/17 cells transfected with 1) HOIL1 and c-Flag pcDNA3 vector, 2) HOIL1 and TRAF1 FL, 3) HOIL1 and TRAF1-ΔCC, 4) HOIL1 and TRAF1-S283A and 5) HOIL1 and TRAF1-H281E/S283E followed by IB with HOIL1-specific antibody and a light-chain-specific anti-mouse-horseradish peroxidase. (d) IB with TRAF2-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase.

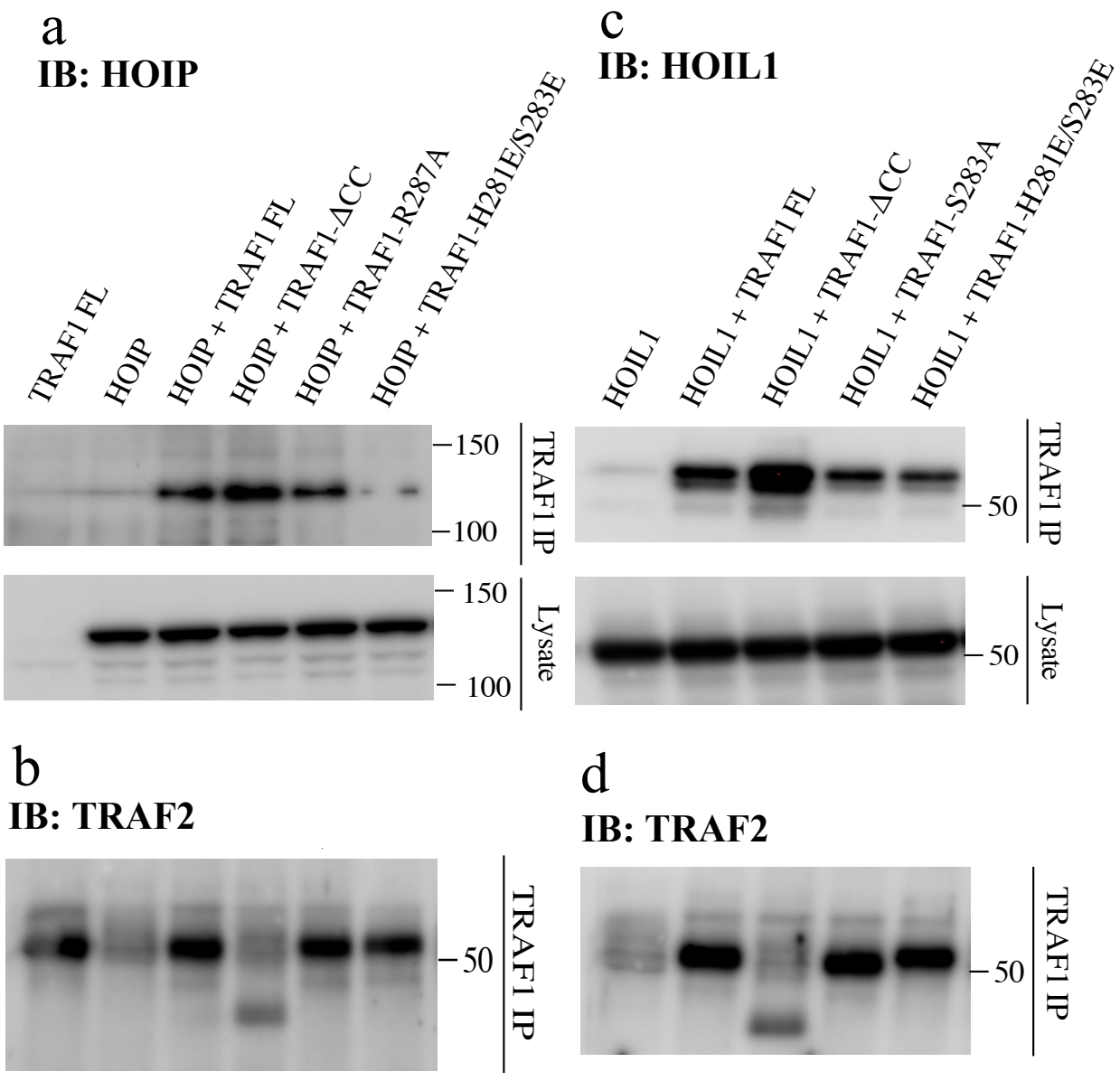
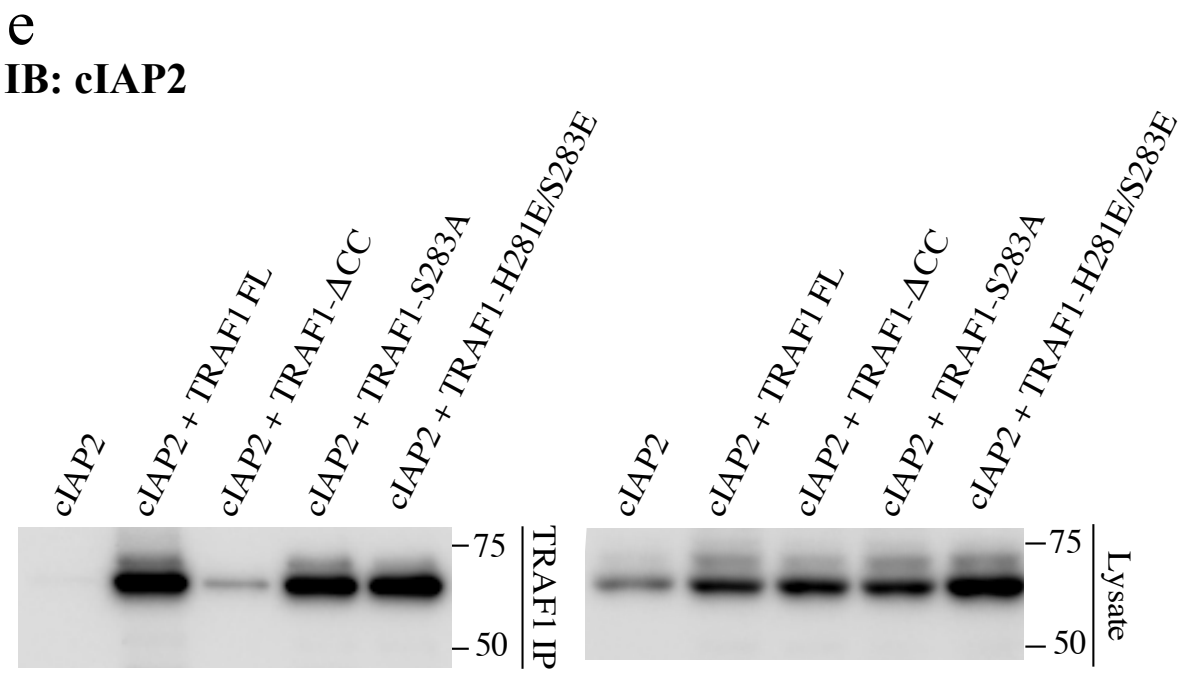


Figure 16. (e) IP with TRAF1-specific antibody of HEK 293T/17 cells transfected with 1) cIAP2 and c-Flag pcDNA3 vector, 2) cIAP2 and TRAF1 FL, 3) cIAP2 and TRAF1-ΔCC, 4) cIAP2 and TRAF1-S283A and 5) cIAP2 and TRAF1-H281A/S283E followed by IB with cIAP2-specific antibody and a light-chain-specific anti-rabbit-horseradish peroxidase. This experiment was conducted twice.



6.0 Discussion

TRAF1 regulates NF-κB activity through two distinct mechanisms each resulting in completely different outcomes downstream of various receptors and cell types. By associating with TRAF2 to form a heterotrimer complex, TRAF1 helps recruit cIAP2 and enhances K63-ubiquitination of NEMO leading to NF-κB activation in lymphocytes (37). In contrast, TRAF1 associates with LUBAC components and prevents linear ubiquitination of NEMO leading to decreased NF-κB activation in monocytes/macrophages (69).

The opposing roles of TRAF1 are both important in regulating the immune response. Induction of NF-κB is necessary to initiate the innate immune response and help the body defend itself against foreign pathogens, however, overactivation of these pathways can lead to the development of autoimmune diseases such as rheumatoid arthritis, multiple sclerosis, type I diabetes mellitus, systemic lupus erythematosus and atherosclerosis (42).

Interestingly, TRAF1 has been associated with an increased risk for RA as both monocytes and T-cells from healthy subjects with a disease-associated SNP express lower levels of TRAF1 leading to a heightened state of inflammation, a major characteristic of RA (69). Since RA is a multi-faceted disease that affects both arms of the immune response and a myriad of signaling pathways, understanding the role of TRAF1 in RA requires dissecting its opposing effects on NF-κB activation and studying them individually, in-vivo. Hence, the purpose of this project was to isolate the distinct roles of TRAF1 by determining the exact protein-to-protein interaction between TRAF1 and LUBAC components.

Using co-immunoprecipitation, we first verified that TRAF1 interacts with HOIL1 through its MATH domain. We then identified the region of the MATH domain involved in the interaction with HOIL1 and HOIP, amino acid residues 277-288. Although both HOIL1 and HOIP interact with TRAF1 through their NZF domain, we found the interaction occurs at

different residues. The interaction between HOIL1 likely occurs with histidine residue 281 and serine residue 283 and the interaction with HOIP occurs with arginine residue 287. We also found that it may be more advantageous when creating the TRAF1 mutant to substitute residues 281 and 283 with an aspartic acid instead of an alanine. Furthermore, it was important to confirm these residues did not interfere with TRAF1's interaction with cIAP2 and TRAF2, as the ultimate goal is to create a TRAF1 mutant that maintains its positive role in inducing NF-κB activation downstream of TNFR family members. It was shown that TRAF1-H281E/S283E interferes with the interaction with both HOIL1 and HOIP but retains its interaction with cIAP2 and TRAF2.

Although we were able to identify, through co-immunoprecipitation, that the interaction of TRAF1 and LUBAC occurs between residues H281, S283 and R287, we were unable to verify through a functional assay that mutating these residues nullifies TRAF1's role in decreasing NF-κB activation. We were working on developing a dual-luciferase reporter assay in which both HOIL1 and HOIP were overexpressed along with a Firefly and Renilla luciferase in 293T/17 cells. Since HOIL1 and HOIP have been shown to induce NF-κB activation on their own, we were expecting an induction of the Firefly luciferase, as it is inducible by NF-κB. By overexpressing TRAF1 along with the aforementioned plasmids, we were expecting a reduction in Firefly luciferase induction. Although we did see these results, when we substituted TRAF1 with either TRAF1 mutant, TRAF1-ΔMATH, positive control or TRAF1-ΔCC, negative control, the results did not show a clear trend and were arbitrary. We hypothesized this was likely due to the inconsistency in Renilla luciferase values for each condition.

Another component to this project was to knockout TRAF1 from THP-1 cells, a monocytic cell line, in order to study the effects of these TRAF1 mutants on NF-κB activation in-vitro. We implemented the CRISPR-Cas9 system, which comprised of transfecting cells with

the Cas9 endonuclease and annealed guides. These guides were designed to target sequences of the TRAF1 locus with maximum knock-out efficiency while minimizing off-target effects. A total of 3 guides were designed, one from Invitrogen's CRISPR design tool, one from IDT's CRISPR design tool and one using the CRISPOR program. Since THP-1 cells are immune cells they do not readily take up foreign DNA, hence, we had substantial difficulty transfecting the Cas9 and annealed guides into these cells. We initially tried lipofectamine but were unsuccessful, hence, we are now implementing electroporation in order to introduce the CRISPR-Cas9 system into these cells. Since electroporation involves applying an electrical current to cells in order to increase cell membrane permeability, there is a fine balance of how much voltage can be applied to these cells before cell viability descends rapidly, thus, we are working on optimizing the protocol to find the right balance between cell permeability and viability.

Once we have confirmed through the dual-reporter luciferase assay that our TRAF1 mutant does not inhibit NF-κB activation, the next steps would be to study this mutant's effects downstream of TLR and TNFR family members in TRAF1-knockout THP1 cells. Since 293T/17 cells are not immune cells the effects of this TRAF1 mutant in 293T/17 cells may differ in an immune cell line such as THP1 cells, hence, it is important to corroborate these findings in THP1 cells before creating a knock-in mouse possessing this TRAF1 mutant and studying its effects in inflammatory diseases such as RA.

7.0 Future Directions

Although we were successful in discerning the sites of interaction between TRAF1 and LUBAC components, HOIL1 and HOIP, in order to generate a mutant that completely abrogates the interaction between LUBAC requires combining these mutations into one mutant and examining how the interaction is affected. In addition, we only showed how the interaction between TRAF1 and LUBAC components is affected when amino acid residues 281, 283 and 287 are mutated, but still require to show the functional repercussions of mutating these residues on NF-κB activation.

The next steps for this project would be to examine how these mutations affect the interaction of mouse TRAF1 and LUBAC components. We predict these sites of interaction or neighbouring sites will hold true in the mouse model. Once a TRAF1 mutant has been designed that does not interact with LUBAC and negatively regulates NF-κB activation downstream of TLR family members, but maintains its interaction with cIAP2 and TRAF2 and positively regulates NF-κB activation downstream of TNFR family members, we will be able to assess the in-vivo role of TRAF1 in knock-in mice based on the mutations and then do models of RA or other inflammatory diseases.

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